Composition and functioning of iron-reducing communities in two contrasting environments, i.e. a landfill leachate-polluted aquifer and estuarine sediments

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Cover illustration: 'SEA'- To remember the days at VU, The Netherlands as shown on my computer screen; to link my research work-in the Scheldt estuary; to record the first view of sea in my life.

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Composition and functioning of iron-reducing communities in two contrasting environments, i.e. a landfill leachate-polluted aquifer and estuarine sediments

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and

Yiqun and Jiayi
My parents and the people
Who generously provided supports

Chapter 1

General introduction

Background on iron reduction

Iron is the fourth most abundant element in the Earth's crust (24) and, more specifically, the most abundant electron acceptor in soils (100) and in some marine sediments (102). Two important forms of iron, i.e. ferric (Fe (III) iron and ferrous iron can be interconverted by a redox reaction, i.e.

$$Fe\ (III) + e^- \rightarrow Fe\ (II)$$

For a long time, iron reduction in sediment environments was considered to be primarily an abiotic process (25, 29, 120). It was reported that the rate of iron reduction was solely determined by pH and/or redox potential, and not by microbial activity (64, review). Iron is reduced non-enzymatically by a variety of substances, including sulfide in sulfate-reducing intertidal mud flats (42), humic acid (itself reduced by microbial activity) and other extracellular quinones and hydroquinones (71, 72, 114), formate (24) and some other metabolites of a number of microorganisms (29).

However, the non-enzymatic reduction of iron is not considered the dominant mechanism: Much of the reduction of iron in the soils is or has been carried out by microorganisms, where microbial iron reduction is mediated by enzymes. For instance, iron reduction disappeared when iron-reducing bacteria were separated from Fe (III) oxides (84). In some organisms nitrate reductase was implicated as a catalyst responsible for the reduction of Fe (III) (97, 98). Respiration is associated with the bacterial cytoplasmic membrane. Research on *Shewanella putrefaciens* MR-1 showed that c-type cytochromes localized in the cells' outer membrane linked insoluble oxides to the electron transport system. When grown anaerobically, membranes of these and other organisms are strongly enriched in cytochromes. This cytochrome distribution could play a key role in iron and manganese respiration (85, 86). In *Geobacter sulfurreducens* iron (III) reductase was found to reside in the outer membrane (28). The gene encoding the secretion of a protein locating to the outer membrane and involved in iron reduction in *S. putrefaciens* has been identified, and provided the first genetic evidence of a connection between dissimilatory metal reduction and so-called type II protein secretion. Additional biochemical evidence indicates the outer membrane localization of *S. putrefaciens* proteins involved in anaerobic respiration with Fe (III) or/and Mn (IV) as electron acceptor (22). Lovley et al. first discovered that microorganisms could oxidize organic compounds all the

way to carbon dioxide using Fe (III) as sole electron acceptor, thereby showing the direct enzymatic route for iron reduction (75). This process, in which microorganisms transfer electrons to external ferric iron, reducing it to ferrous iron without assimilating the iron, has been defined as dissimilatory Fe (III) reduction. Iron reduction that can conserve free energy from electron transfer and use that to drive microbial growth is preferably called ferric respiration (65).

Achievements of the research on iron reduction hold promise for our daily life in the near future. Knowledge on the principles of natural attenuation of pollutants has begun to help us to enhance *in situ* bioremediation, which often occurs under iron-reducing conditions, and to remove pollutants from environments. Electrons produced during the iron-reducing process by Geobacters (6, 7) can be harvested and transferred into electricity. This may be a novel approach to generate power through microbial respiration while clearing contaminated environments. The ability of *G. sulfurreducens* to transfer electrons from the cell surface to the surface of Fe (III) oxides via pili as biological nanowires, might be also useful for other bioengineering of novel conductive materials (103).

While the geochemical and ecological importance of the enzymatically-mediated reduction of iron oxyhydroxides in subsurface systems is widely recognized, it is still poorly understood. The objective of the research described in this thesis is to advance the understanding of the geochemical, environmental and ecophysiological factors that determine the activity of the Fe (III)-reducing microbial populations in the subsurface. This research aims at gaining mechanistic insight into iron reduction, more specifically, into understanding the composition of iron-reducing communities, and their functioning in two contrasting types of environments, i.e. an aquifer polluted by a neighbouring landfill and estuarine sediments.

In this chapter, I will present an introduction to important aspects of iron reduction. I shall address: (i) the global importance of enzymatic iron reduction, (ii) where and how iron reduction occurs, (iii) which microorganisms are capable of reducing iron oxyhydroxides, (iv) how iron reducers access Fe (III) oxides, (v) the relationship between bioavailability of Fe (III) oxides and iron reduction, (vi) the electron donors usually encountered in subsurface and (vii) the microbial communities involved in iron-reduction.

The importance of enzymatic iron reduction

Fe (III) reduction may have been amongst the earliest forms of microbial respiration (24). Studies on *Archaea* and *Bacteria* that are most closely related to the last common ancestor suggest that Fe (III) rather than sulphur has been the first external electron acceptor in microbial metabolism (128). These *Archaea* and *Bacteria* are located low in the phylogenetic tree and are all capable of reducing Fe (III) with electrons taken from hydrogen. Molecular hydrogen (i.e. H_2) and Fe (III) are thought to have been present in elevated amounts on prebiotic Earth (130), due to the accumulation of Fe (III) from hydrothermal fluids, due to resulting high-intensity UV radiation of Archaean seas

containing dissolved Fe (II), and through the accumulation of hydrogen from geotectonic activity (12). Thus, conditions prior to the evolution of life favored the development of hydrogen-oxidizing and Fe (III)-reducing microorganisms. Comparable conditions are found on Mars. Meteorites of Mars revealed a close association of two minerals, iron oxides called magnetite and iron sulfides (http://curator.jsc.nasa.gov/antmet/marsmets/life.htm). These minerals can be formed in the secondary mineralization by bacteria on Earth [27, (53, review), 54(review)]. This might be considered as a trace of life on Mars.

Research on iron reduction is rapidly increasing. In different fields such as geology, geochemistry, and in particular, geomicrobiology, insight has been gained into the role of microorganisms in iron-reducing process. In addition to being the earliest microbial respiration process on Earth, metal reduction also contributed to metal deposits and carbon sequestration from secondary mineralization as a result of formation of insoluble forms containing metal elements such as, Mn, Co, Al, Cr (9), U (2, 23), As (2, 23, 36). Some metal elements are found co-precipitated with C, P or S and entrapped in the environment in solid forms such as siderite ($FeCO_3$), vivianite [Fe_3 (PO_4) $2.8H_2O$], green rust precipitates $\{(FeII)_{6-x}\ FeIII_x\ (OH)_{12}]^{x+}\ [A^{2-})_{x/2}.yH_2O]^{x-}\}$. Studies on metal-deposition and co-precipitation help in understanding the mechanisms of element sequestration in the environments.

From the perspective of human society, it is of great interest that in strictly anaerobic, polluted environments the process of iron reduction is associated with the degradation of organic matter. Indeed, iron-reducing Geobacters dominated a petroleum-contaminated aquifer (108). Toluene, phenol, and para-cresol as well as TNT and other (poly) nitroaromatic compounds (37), were oxidized anaerobically by iron-reducing microorganisms (75). Anaerobic degradation of monobrominated phenols and benzoic acids by microorganisms enriched from marine and estuarine sediments was observed under iron-reducing conditions (83). In addition, benzene, the monoaromatic compound that is most difficult to degrade, can be oxidized to CO_2 under iron-reducing conditions by microorganisms (48).

Occurrence of iron reduction processes

Iron-reducing processes under anaerobic conditions have been observed in a variety of environments. Most studies related to iron reduction have been conducted in marine sediments (19, 35, 50) and fresh water sediments (26, 55, 127, 129). The phenomenon of iron reduction has been found to be ubiquitous: It occurs in landfill leachate-polluted aquifers (1, 39, 124), in niches such as rhizospheres (14, 56, 131), and in waste water (95).

In subsurface environments, nitrate, Mn (IV), Fe (III), sulfate and CO_2 are potential terminal electron acceptors for the anaerobic oxidation of organic matter (69). It is assumed that a TEAP (terminal electron acceptor process) will only occur when the

Gibbs free energy drop for the reaction exceeds a minimum threshold value (7 kJ/mol H_2)(5, 43, 67, 70, 81, 113). In aquifers contaminated with petroleum or other organic pollutants such as occur in as landfill leachate, there is a distinct zonation of TEAPs, going from high to low Gibbs-energetic yield: nitrate reduction > Mn (IV) reduction >Fe (III) reduction > sulfate reduction>methanogenesis (15, 123, 125).

Iron-reducing microorganisms appear to outcompete sulfate reducers and methanogenic microorganisms for electron donors, i.e. for the organic matter. Indeed, Fe (III) reduction takes place prior to sulfate reduction (64). A competitive mechanism causing inhibition of sulfate reduction and methane production in the zone of ferric iron reduction in sediments might be the explanation: much lower concentrations of hydrogen and acetate in sediments were found when Fe (III) was the predominant terminal electron acceptor, as compared to sediments in which sulfate reduction and methanogenesis were the predominant electron accepting processes (77). Such low concentrations of electron donors could correspond to thermodynamically unfavorable conditions for sulfate reduction certainly as compared to Fe (III) reduction (119), unless the amount of bioavailable Fe oxides is too low to start or sustain iron reduction.

Although there is a sequence of preference for TEAPs, overlap of iron-reduction, sulfate-reduction and methanogenesis zones has also been observed, probably due to the influence of pH, redox species concentrations, bioavailability of Fe (III) oxides (15, 16), fermentation of organic compounds and release of reduced redox species (Mn (II), Fe (II), H_2S, CH_4) (123).

Dissimilatory iron-reducing microorganisms

Enzyme-mediated iron reduction is conducted by microorganisms that are able to synthesize the relevant enzymes. These microorganisms are able to dissimilate Fe (III), inhabit diverse environments and are phylogenetically quite diverse. Based on their metabolic capacities, dissimilatory Fe (III)-reducing microorganisms are divided into two groups. The first group of iron reducers does not conserve Gibbs energy from the process of iron reduction in order to support growth, but uses Fe (III) as an electron sink only. Iron-reducing microorganisms from the second group can conserve Gibbs energy from iron reduction and apply this to support growth.

With the former group of iron reducers a substantial fraction of the electron equivalents extracted from the electron donors is recovered in fermentation products and hydrogen. Less than 5 % of the reducing equivalents are transferred to Fe (III) (66, 78). The ability to direct part of the electron donor to acetate production by using Fe (III) as an electron sink then results in additional ATP formation, via substrate-level phosphorylation. Gibbs energy from Fe (III) reduction is a by-product and is not necessary for the growth of these microorganisms (68). This group consists of fermentative Fe (III) & Mn (IV)-reducing microbial species from genera all across the three domain of Life (i.e. *Bacteria, Archaea* as well as *Fungi*) (cf. Table 1) (68).

The microorganisms that transduce Gibbs energy from Fe (III) respiration via oxidative phosphorylation to new biomass are phylogenetically diverse. All members of *Geobacteracea*, consisting of the genera *Geobacter*, *Desulfuromonas*, *Desulfuromusa* and *Pelobacter*, and affiliated with the delta (δ) subclass of Proteobacteria, are capable of harvesting Gibbs energy from Fe (III) or Mn (IV) reduction coupled to organic oxidation to support their growth (65.). As table 1 shows, the δ subclass of Proteobacteria contains the largest division of iron reducers known to date. Most currently known iron respiring microorganisms are Proteobacteria.

Many sulfate-reducing microorganisms (see Table 1) have been found to be able to grow anaerobically in the same way as iron reducers, i.e. using the same electron donors and reducing Fe (III). However, not all sulfate reducers have been shown to grow with Fe (III) as the sole electron acceptor (73). Thus, Fe (III) reduction conducted by sulfate reducers is not considered a growth-supporting process. This may be a strategy for sulfate-reducing microorganisms to survive and thrive in an environment in which sulfate is present but in a limited amount, as sulfate reducers can use Fe (III) when sulfate is depleted. Alternatively, Fe (III) reduction by sulfate reducers may be advantageous to enhance competition conditions for sulfate reduction by depleting Fe (III)(68). However, the mechanism of iron reduction conducted by sulfate reducers is not well investigated yet.

Fe (III) reducers have not only been found among *Bacteria* but also among the *Archaea*. Iron reduction by iron reducers from *Archaea* is usually conducted at high temperatures. *Archaeoglobus fulgidus, Methanococcus thermolithotrophicus, Methanopyrus kandleri, Pyrococcus furiosus*, and *Pyrodictium abyssi*, reduce Fe (III) and oxidize hydrogen at 90 ^{o}C (128). *Pyrobaculum islandicum* reduce Fe (III) and Mn (IV) at 100 ^{o}C (47) and hyperthermophilic iron-reducing bacteria strain 121 grows at temperatures as high as 121 ^{o}C, a temperature which is used for sterilization of microbe-cultivation media (46). Two hyperthermophiles, i.e. *Ferroglobus placidus* and *Geoglobus ahangari*, can reduce Fe (III) with the oxidation of acetate at 85 ^{o}C. *Ferroglobus placidus* is able to degrade aromatic compounds coupled to Fe (III) reduction (121).

Most iron-reducing microorganisms are mesophilic, growing well around neutral pH values (68). However, enzymatic iron reduction has been documented in acid mine environments (3, 17, 44). Microbes engaging in this process were also recovered from acidic environments, such as iron-reducing acidophilic thermophile – strain SJH (45), *Acidiphilium cryptum* JF-5 (57), *Acidiphilium spp.* (44). *Thiobacillus ferroxidans* is capable of growing anaerobically with sulfur as electron donor and Fe (III) as the electron acceptor (21). *Thisobacillus thiooxidans* and *Sulfolobus acidocaldarius* have similar capabilities (10).

As stated above, most iron-reducers are found among the *Geobacteraceae*. Iron reducers from *Geobacteracea* are strict anaerobes and isolated from subsurface environments. However, it has been reported that *G. sulfurreducens* can tolerate oxygen

Table 1. Affiliation of dissimilatory Fe (III) microorganisms.*

Bacteria			
iron respiration		as a sink (fermenting)	
Genera	Phylum (class)	Genera	Phylum (class)
Ferribacterium	Proteobacteria (β)	*Actinomucor*	Proteobacteria (γ)
Rhodoferax	Proteobacteria (β)	*Aerobacter*	Proteobacteria (γ)
Aquaspirillum	Proteobacteria (β)	*Pseudomonas*	Proteobacteria (γ)
Shewanella	Proteobacteria (γ)	*Escherichia*	Proteobacteria (γ)
Ferrimonas	Proteobacteria (γ)	*Serratia*	Proteobacteria (γ)
Aeromonas	Proteobacteria (γ)	*Vibrio*	Proteobacteria (γ)
Pantoea	Proteobacteria (γ)	*Paracolobactrum*	Proteobacteria (γ)
Pelobacter	Proteobacteria (δ)	*Rhodobacter*	Proteobacteria (α)
Anaeromyxobacter	Proteobacteria (δ)	*Thiobacillus*	Proteobacteria (β)
Geothermobacter	Proteobacteria (δ)	*Wolinella*	Proteobacteria (ε)
Desulfuromusa	Proteobacteria (δ)	*Bacteroides*	Bacteroidetes
Geobacter	Proteobacteria (δ)	*Clostridium*	Firmicutes
Desulfuromonas	Proteobacteria (δ)	*Bacillus*	Firmicutes
Sulfurospirillum	Proteobacteria (ε)	*Sulfolobus*	Aquificae
Geospirillum barnesii	Proteobacteria (ε)	**Sulfate reducer**	
Geovibrio	Deferribacteres	Genera	Phylum (class)
Deferribacter	Deferribacteres	*Desulfobacter*	Proteobacteria (δ)
Desulfitobacterium	Firmicutes	*Desulfobacterium*	Proteobacteria (δ)
Bacillus	Firmicutes	*Desulfobulbus*	Proteobacteria (δ)
Geothrix	Acidobacterium	*Desulfovibrio*	Proteobacteria (δ)
Thermoterrabacterium	Gram positive	*Desulfomicrobium*	Proteobacteria (δ)
Deinococcus	Gram positive	*Desulfotomaculum*	Firmicutes
Alkaliphilus	Gram positive		
Thermotoga	Thermotogales		
Thermus	Green nonsulfur		
Archaea			
iron respiration		as a sink (fermenting)	
Genera	Phylum (class)	Genera	Phylum (class)
Archaeoglobus	Euryarchaeota	*Sulfolobus*	Crenarchaeota
Methanococcus	Euryarchaeota		
Methanopyrus	Euryarchaeota		
Pyrococcus	Euryarchaeota		
Pyrodictium abyssi	Crenarchaeota	***Fungi***	
Geoglobus	Euryarchaeota	Genera	Phylum (class)
Ferroglobus	Euryarchaeota	*Fusarium*	Eukaryota
Pyrobaculum	Crenarchaeota	*Alternaria*	Eukaryota

** Microorganims are divided into two groups based on their metabolic behavior for iron reduction: underlined genus contains species that are capable of conserving Gibbs energy and use that to support their growth, using Fe (III) merely as an electron a sink. The others are not known to be capable of this Gibbs-energy transduction and must obtain Gibbs energy from the iron reduction process itself. 'fermenting' refers to the absence of electron transfer chain activity; phosphorylation being substrate-level only.*

thereby surviving in diverse environments (59). Members of the *Shewanella* genera and *Thermus SA-01*(49) are able to grow facultatively with iron. *Shewanella* species are recovered from shallow sediments, where lactate or large ranges of organic compounds are present.

Current evidence demonstrates that metal reduction is a widespread characteristic in *Bacteria*, *Archaea* and *Fungi*. New iron-reducing microorganisms are frequently reported in the literature.

Mechanisms of iron reduction

The precise mechanisms by which iron-reducing microorganisms access Fe (III) oxides are still poorly understood. Three mechanisms have been proposed on how iron-reducers utilize the insoluble iron oxides (Figure 1).

First, direct contact between iron-reducing microorganisms and Fe (III) oxides (illustrated in square A in Figure 1) is proposed to involve electrostatic forces. This direct contact enables possible functioning of the Fe (III)-reductase, which is primarily located in the outer membrane of Fe (III)-reducing microorganisms such as *S. putrefaciens* (85, 87), *G. metallireducens* (31) and *G. sulfurreducens* (28). This direct contact was proposed as being necessary for iron reduction in systems, in which electron shuttling compounds and chelators (to be discussed below later) were lacking (68). *G. sulfurreducens* (82) and *G. metallireducens* (13) express flagella and pili specifically, and characteristic of *Shewanella oneidensis* MR-1 (4) when growing on insoluble Fe (III) and Mn (IV) oxides bestowing these organism with chemotaxis towards Fe (II) and Mn (IV) in the absence of soluble electron acceptors or chelators.

Second, some dissimilatory Fe (III)-reducing microorganisms are capable of secreting electron shuttling compounds, which are used for electron transfer between Fe (III) oxides and cells. These include extracellular compounds from *Geothrix fermentans* (92), the phenazines produced by *Pseudomonas chlororaphis* (34), the siderophore produced by *S. putrefaciens* (58), and melanin from *S. algae* (122) (cf. square B, Figure1). *G. sulfurreducens* was first reported to produce a 9.6 kDa c-type cytochrome capable of transferring electrons extracellularly to insoluble iron hydroxides (115). However, further research demonstrated that the 9.6 kDa cytochrome was not the dominant extracellular c-type cytochrome (63). Iron-reducers do not depend on the ability to produce electron shuttles themselves in order to use this mechanism in iron reduction. Humic acid and other extracellular quinones (71, 72) may constitute electron shuttles promoting iron reduction by Fe (III)-reducing microorganisms. Humic substances are ubiquitous and abundant in soils and sediments and originate from degradation of plants, animals and microorganisms (80). Under strictly anaerobic conditions, many Fe (III)-reducing microorganisms are capable of using humic acid as an electron acceptor (18).

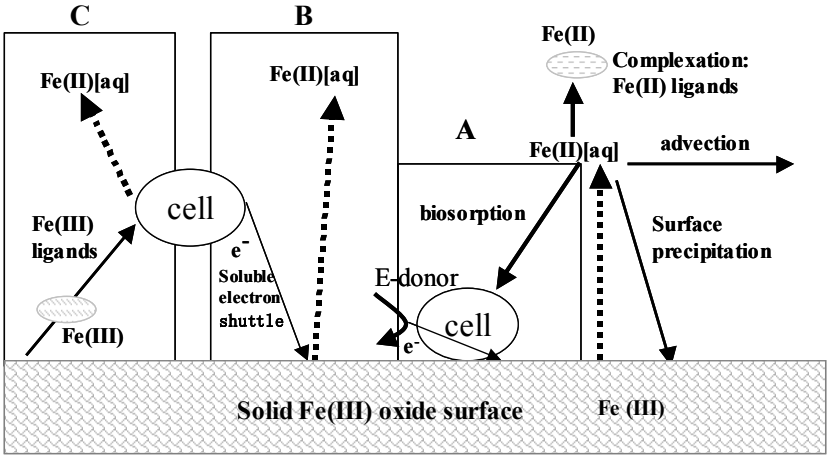

Figure 1. *Scheme showing the proposed mechanisms of iron reduction and possible interactions between microorganisms and iron oxides. Square A, B and C illustrate the mechanisms of iron reduction by means of direct contact by cells, extracellular electron shuttles, and chelation respectively. Dashed arrows and texts show the flow direction of Fe (II) in different processes, biosorption, complexation and precipitation. [aq] stands for Fe (II) being in aqueous phase.*

Thirdly (cf. square C in Figure 1), ligands or chelators present in the environment can chelate Fe (III), and then the chelated and solubilized iron (III) can be accessed by the microorganism. Examples include organic acids such as citrate, oxalate, NTA (nitrotriacetic acid) and the inorganic acid phosphate. Some iron reducers are known to produce chelators: extracellular compounds secreted by *S. alga BrY* (93) and *Geothrix fermentans* (92) can enhance the solubility of Fe (III) oxides.

As discussed above, different mechanisms are found in different microbial species and one microorganism can have more than one mechanism enabling it to face changing conditions. Table 2 summarizes mechanisms applied by various iron-reducing microorganisms.

Availability of Fe (III) oxide

Most terminal electron accepting processes involve soluble acceptors such as nitrate and sulfate. Iron reduction is an exception as it deals with the Fe (III) oxides that are insoluble in water. Thus, the properties of the iron oxides may influence the occurrence and rate of iron reduction. These include their surface area, their structure, the presence of chelators as well as Fe (II) sorption onto Fe (III) oxides and/or Bacteria (cf. below). Indeed, the solubility of Fe oxides has been shown to be important for mineral surface-associated cell activity (8, 90). The surface area of Fe (III) oxides determines the bioavailability of Fe (III) oxides (60, 106, 132). Based on their structure, two types of Fe oxide are distinguished. The crystalline iron oxides, such as hematite (α-Fe_2O_3), goethite (α-FeOOH), akaganetite

(β-FeOOH), lepidocrocite (γ-FeOOH), magnetite (γ-Fe_2O_3), are more difficult to be used by microorganisms. Poorly crystalline iron oxides, which are the primary Fe (III) forms in aquifer sediments (76), i.e., amorphous oxide such as 2-line ferrihydrite (the '2-line' referring to its X-ray diffractogram), are utilized much more readily by iron-reducing microorganisms.

Table 2. Summary of proposed mechanisms to access Fe (III) oxides as applied by iron-reducing microorganisms

	Proposed mechanisms of iron reduction			
microorganism	Electron shuttle	Fe (III) chelator	Chemotaxis	Reference
S. oneidensis	quinone	Not reported	Yes	(4, 94)
S. algae	melanin	Not reported	No	(122)
S. putrefaciens	Not reported	siderophore-putrefactin	No	(58)
Geothrix fermentans	qQuinone	extracellular compound	No	(92)
Pseudomonas chlororaphis	phenazines	No	Not reported	(34)
G. metallireducens	No	No	flagella, pili	(13, 91)
G. sulfurreducens	No	Not reported	flagella, pili	(82)

The bioavailability of amorphous ferrihydrite was more than twice that of crystalline, high surface area (HSA) goethite, ten times that of crystalline low surface area (LSA) goethite, and 20 times that of crystalline hematite (33). Species of heterotrophic *Shewanella*, a genus in the γ-*Proteobacteria*, have often been used as model strains in Fe (III) reduction studies (11, 30, 32, 60, 61, 116). In studies with *S. putrefaciens* (8) and *G. metallireducens* (Chapter 6 of this thesis), the initial reduction rate of amorphous ferrihydrite was found to be higher than that of crystalline iron oxides. The initial rate and long-term extent of reduction of a range of synthetic iron oxides by *S. alga* were linearly correlated with the oxide surface area. Therefore, the rate and extent of microbial iron oxide reduction appeared to be controlled by the surface area and available site concentration of the solid phase (106).

Different chelators pose different constraints on iron reduction. Fe (III) chelated by citrate is readily reduced. Iron reduction rates were very low when the stronger chelators Tiron and EDTA were used (32). Natural organic matter (NOM) and AQDS (anthraquinone 2,6-disulfonate, humic acid analog) were observed to enhance hematite reduction by shuttling electron and/or by chelation.

Iron reduction is also influenced by its product Fe (II) (see also Figure 1). The primary factor controlling the nature of the secondary mineralization of Fe (II) appears to be the rate and extent of ferrous supply, and its surface reaction with the residual oxide and other sorbed iron (132). Accumulation of Fe (II) coatings on Fe (III) oxide and FeRB (iron-reducing bacteria) surfaces causes the reaction to slow down due to a decreased thermodynamic driving force (111), and may inhibit enzymatic Fe (III) oxide reduction (in

the absence of soluble electron shuttles and/or Fe (III) chelators) (104). The sorption of Fe (II) and /or Fe (III) to iron oxides and bacteria, which gives rise to precipitation, leads to a decrease in iron reduction rate as the surface of iron oxide available to the bacteria becomes smaller. Removal of biogenic Fe (II) allowed a dramatic increase in the extent of Fe (III) oxide reduction and associated bacterial growth in a continuous-flow column reactor (105). Fe (II) complexation appeared to alleviate the suppression of hematite reduction rate caused by accumulated Fe (II) in the system (109, 110). In natural sediments, highly reduced AQDS may compete more effectively with Fe (II) for hematite surface sites and decrease the negative impact of Fe (II) on iron reduction (27).

Electron donors in subsurface environments

The type of electron donor also influences the rate of reduction of Fe (III) oxide reaction. Although complex organic matter can serve as electron donor in anoxic environments (64), acetate is the major intermediate of fermentation and functions as an electron donor for iron reducers (79). Hydrogen is another important electron donor for iron reduction in subsurface sediments. At steady-state, H_2 concentrations depend primarily on the physiological characteristics of the microorganisms consuming H_2 (74).

Molecular microbial ecology and iron-reducing microbial communities

Microbial ecology has rapidly advanced over the last two decades through the introduction of molecular techniques. Molecular analyses of microbial communities based on amplified 16S rRNA gene assist in obtaining information on not yet-culturable microorganisms in iron-reducing environments, which usually outnumber culturable microbes. Phylogenetic information on iron-reducing enrichments and natural environments indicated that iron-reducing microorganisms are quite ubiquitous and diverse. In the subsurface, various clones affiliated with *Geobacteraceae* appeared to dominate a wide variety of environments, i.e. aquatic sediments after stimulation of iron reduction (117), a petroleum-contaminated aquifer (108), metal-polluted freshwater lake sediments (2, 20, 38, 41) and a landfill leachate-polluted aquifer. In the latter around 25 % of the phylotypes were *Geobacter spp*. (107): Geobacters appear to play an important role in organic matter degradation and in heavy metal immobilisation.

Phylotypes from other genera were also encountered in iron-reducing environments. From 860 m below the surface, sequences closely related to *Thermoanaerobacter ethanolicus* or *T. kivui* were recovered (133). Bacterial 16S rDNA fragments from DGGE gels affiliated with *Clostridium*, *Thiobacillus aquaresulis, Denitrobacterium detoxificans, Bacillus infernos, Actinomycete, Bacteroides distasonis, Erysipelothrix, Cystobacter* and uncultured *Eubacterium*, were retrieved during experiments in which iron reduction was stimulated (117). In addition to known Geobacters, sequences closely related to the Fe (III) reducer *Geothrix fermentans* were found in benzene-degrading enrichments and sediments (108). Rather than the more often

encountered *Geobacter* and *Shewanella*, iron-reducing microorganisms capable of producing spores *Anaeromyxobacter*, *Paenibacillus* and *Brevibacillus spp* (affiliated with low G+C Gram positives) were representatives of acidic subsurface environments contaminated with uranium (96, 99). The update research has shown that sequences closely related to *Proteobacteria* constituted a large proportion of retrieved phylotypes in iron-reducing enrichments and in sediments associated with pollutant degradation (95, 107). However, so far, there is not much information on iron-reducing microbial communities as compared to what is known about sulfate-reducing microbial communities (51, 52, 62, 88, 89, 101, 112).

This study

The objective of the research described in this thesis is to advance the mechanistic understanding of how geochemical, environmental and ecophysiological factors control the activity of Fe (III)-reducing microbial populations in the subsurface. Therefore, culture-dependent (batch and retentostat cultivation) and culture-independent (16S rRNA gene based techniques) approaches are employed to investigate community structure, phylogenetics and physiology of iron-reducing bacteria populations in two contrasting iron-reducing environments.

Research locations

One of the sites studied is the Banisveld landfill, located 5 km southwest of Boxtel, The Netherlands (NL), EU. Unlined landfilling primarily with household refuse occurred in a 6 m deep sand pit between 1965 and 1977. The aquifer consists of an 11 m thick layer of fine to coarse unconsolidated sands positioned upon less permeable clay and peat deposits alternating with sandy layers. Groundwater flow (approximately 4-10 m/year) is directed northeast to north towards a nature reserve, which is a habitat for a rare oligotrophic ecosystem. The biogeochemical processes have been surveyed and the leachate composition in the flow direction of the landfill plume has been determined (124). Microbial physiology has been profiled with respect to pollution and substrate utilization using BIOLOG plate studies (107). Natural attenuation has been confirmed in this studied site; however, the relationship between (i) microbial community structure, and (ii) hydrochemistry, (iii) pollutant degradation, and (iv) the functioning of the corresponding microbes are unknown.

Microbial iron reduction was also studied at two intertidal marsh sites, i.e. ‘Appels’ and ‘Waarde’, both in the Scheldt estuary in Western Europe. The Appels site, located 127 km upstream from Vlissingen, NL, EU in the freshwater estuary, is situated in the part of a river meander that surfaces at low tide. The Waarde site is located 40 km upstream from Vlissingen. It is a brackish part of the lower estuary, known as the Western Scheldt. Those two sites are situated in a heavily polluted estuary that drains water from

the areas around the cities of Antwerp and Brussels, which rank among the most densely populated areas in the world. Therefore, they have been highly influenced by anthropogenic pollution from agricultural, municipal and industrial activities (118). Also, the different salinities at the two sites affect the aquatic ecosystem. A discrete iron-phosphorus (Fe-P) mineral complex is predominant in the sediments of both sites (40). Fe (III) reduction by sulfide was observed in freshwater estuaries (126) and the Scheldt estuary (134). We expected the existence of iron reduction that is directly mediated by microorganisms, at both sites, although abiotic reactions might also be relevant.

Thesis outline

Chapter 2 of this thesis deals with the relationship between microbial community composition and the presence of pollutants, as well as with the type of redox processes that are responsible for pollutant degradation in the leachate-polluted aquifer near Banisveld. The microbial community structure was analyzed by DGGE (denaturing gradient gel electrophoresis) analysis of *Archaea* and *Bacteria* communities in 29 groundwater samples. Properties of sediment-attached communities and groundwater communities were compared for 5 sediment samples and corresponding groundwater samples. Sequence data were obtained to determine the composition and distribution of microbial species in different parts of the aquifer and correlated to level of pollution and occurrence of redox processes.

Chapter 2 shows that there were differences in terms of microbial community structure between polluted and clean parts of the aquifer. *Geobacter spp*. dominated the iron-reducing polluted part of the plume (107). These findings led to the research presented in Chapter 3, which focuses on the relationships between *Geobacter spp.* and hydrogeochemistry (pollution and redox process). Chapter 3 describes and discusses the relations between *Geobacter* population structure, the degree of pollution and the type of electron acceptor (iron or nitrate), and whether or not the presence of specific *Geobacter spp*. was related to degradation of the pollutants.

Results from culture-independent research (Chapter 2, 3) indicated that the iron-reducing *Geobacter spp.* play a role in pollutant degradation near the aquifer beneath the Banisveld landfill. Chapter 4 presents the results of culturing work using different electron acceptors such as Fe (III) citrate, hematite, AQDS (humic acid analog). Such enrichment and isolation should help to understand the functioning of iron-reducing microorganisms in polluted environments. Recovered iron-reducing fractions were tested toward a variety of electron acceptors.

Chapter 5 presents the contribution of iron-reducing microorganisms to iron reduction in another polluted iron-reducing environment, i.e. at the research locations Appels and Waarde in the Scheldt estuary. Specific iron-reducing microorganisms from the genus of *Shewanella*, *Geobacter, Anaeromyxobacteria* and *Geothrix* were detected in various layers of sediments as well as in iron-reducing enrichments. Enrichment and

isolation of iron-reducing microorganisms was performed. The effect of different types of iron oxides and dilution of inocula on microbial composition in enrichments was examined. The composition of the microbial community involved in the reduction of different Fe (III) oxides was determined via 16S rRNA gene analyses. Several iron reducers were isolated as pure cultures. The growth of selected consortia and isolates at various temperatures and pH's was tested as was their flexibility in terms of electron acceptors and donors.

Chapter 6 describes the physiology of *G. metallireducens* under conditions relevant for its natural environment. Low growth rate and low substrate concentrations were obtained by cultivation in a retentostat reactor. Retentostat cultivation was conducted with *G. metallireducens* as a reference strain, which was grown on either a humic acid analog (AQDS) or on acetate as a limiting substrate. This chapter attempts to gain insight into the physiology of Geobacters, which are ubiquitous in subsurface environments. The versatility of *G. metallireducens* grown on AQDS toward alternative electron acceptors, without the need to induce new enzymes, was tested, as well as the iron-reducing capacity for different forms of Fe (III) oxides.

On the basis of the results obtained in the research chapters 2-6, chapter 7 discusses and relates the composition and functioning of the iron-reducing community under natural conditions and puts forth suggestions for *in-situ* bioremediation of aromatics with respect to Geobacters. It projects the composition of iron-reducing communities onto the corresponding environmental settings. It also discusses iron-reduction as one of the determinants of the functioning of ecosystems.

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Chapter 2

Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer

ABSTRACT

Knowledge about the relationship between microbial community structure and hydrogeochemistry (i.e., pollution, redox and degradation processes) in landfill leachate-polluted aquifers is required to develop tools for predicting and monitoring natural attenuation. In this study analyses of pollutant and redox chemistry were conducted in parallel with culture-independent profiling of microbial communities present in a well-defined aquifer (Banisveld, The Netherlands). Degradation of organic contaminants occurred under iron-reducing conditions in the plume of pollution, while upstream of the landfill and above the plume denitrification was the dominant redox process. Beneath the plume iron reduction occurred. Numerical comparison of 16S ribosomal DNA (rDNA)-based denaturing gradient gel electrophoresis (DGGE) profiles of *Bacteria* and *Archaea* in 29 groundwater samples revealed a clear difference between the microbial community structures inside and outside the contaminant plume. A similar relationship was not evident in sediment samples. DGGE data were supported by sequencing cloned 16S rDNA. Upstream of the landfill members of the β-subclass of the class Proteobacteria (β-proteobacteria) dominated. This group was not encountered beneath the landfill, where Gram-positive bacteria dominated. Further downstream the contribution of Gram-positive bacteria to the clone library decreased, while the contribution of δ-proteobacteria strongly increased and β-proteobacteria reappeared. The β-proteobacteria (*Acidovorax, Rhodoferax*) differed considerably from those found upstream (*Gallionella, Azoarcus*). Direct comparisons of cloned 16S rDNA with bands in DGGE profiles revealed that the data from each analysis were comparable. A relationship was observed between the dominant redox processes and the bacteria identified. In the iron-reducing plume, members of the family *Geobacteraceae* made a strong contribution to the microbial communities. Because the only known aromatic hydrocarbon-degrading, iron-reducing bacteria are *Geobacter spp*., their occurrence in landfill leachate-contaminated aquifers deserves more detailed consideration.

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INTRODUCTION

Contamination of groundwater is a serious environmental problem throughout the world as it affects drinking water resources and has an impact on oligotrophic environments. In The Netherlands, an important source of contamination is landfill leachate. In the past, landfilling was performed without the presence of appropriate liners to prevent percolation of leachate into underlying aquifers. Although many old landfills, are closed now, the cessation of landfill operations does not stop chemical release into the environment. Organic compounds originating from household and industrial waste are found in most municipal landfills. Dramatic changes in aquifer geochemistry and microbiology downstream of landfills occur as a result of the high organic load of leachate (11). A sequence of redox zones develops in time and space, as the organic matter is microbiologically degraded and electron acceptors are depleted (11, 29).

Iron reduction and manganese reduction are important redox processes in polluted aquifers (2, 11, 21, 27, 28). Solid iron oxyhydroxides and manganese oxides are reduced, which releases soluble metal species into the groundwater. These metals, together with other reduced species, such as methane, ammonium, and hydrogen sulfide, can pose a threat to drinking water and oligotropic nature reserves (11, 28). Also, pathogenic bacteria might be present in the leachate (11). However, of particular concern is contamination of groundwater by aromatic compounds (especially benzene, toluene, ethylbenzene, and xylene [BTEX]). These compounds are often encountered in landfills (11). Although they account for at most a few percent of the organic matter in leachate, concern about them is related to their toxicity and relatively high solubility. BTEX components are readily degraded under aerobic conditions but are far more persistent under anaerobic conditions (29), which are typical within and downgradient of landfills (11).

It is often difficult and expensive to remediate a subsurface environment. However, despite unfavorable conditions, appreciable anaerobic microbial degradation of BTEX has been observed in landfill leachate-polluted aquifers (1, 34, 44). The ability to predict the potential for natural attenuation and the ability to monitor on-going degradation processes should help limit the number of landfills and aquifers that have to be actively remediated. Thorough knowledge of microbial community structure in polluted aquifers, the capabilities of the microbial populations present, and how these populations affect their environment and vice versa should aid in the development of tools for predicting and monitoring natural degradation. Here, we describe the relationship between hydrogeochemistry and microbial community structure in a landfill leachate-polluted aquifer close to the town of Boxtel, The Netherlands. From this aquifer 29 groundwater samples and five sediment samples were obtained. Chemical analyses were conducted to determine the level of pollution and deduce the principal redox processes. The community structures for members of the *Archaea* and *Bacteria* were determined by denaturing gradient gel electrophoresis (DGGE) (35), and the profiles were statistically compared (42).

For three groundwater samples clone libraries were constructed to obtain more detailed information about the composition of the microbial communities.

MATERIALS AND METHODS

Site description and installation of piezometers. Banisveld landfill is located 5 km southwest of Boxtel, The Netherlands. Unlined landfilling of primarily household refuse occurred in a 6-m-deep sand pit between 1965 and 1977. The aquifer consists of an 11-m-thick layer of fine to coarse unconsolidated sand located on less permeable clay and peat deposits alternating with sandy layers. The direction of the groundwater flow (approximately 10 m/year) is northeast to north towards a nature reserve, which is a habitat for a rare oligotrophic ecosystem. An electromagnetic survey and cone penetration tests revealed the horizontal and vertical location of the leachate (49). In June 1998, this information was used to install a transect consisting of 11 bailer drillings along the direction of groundwater flow (Figure 1). Two or three polyvinyl chloride piezometers with an inside diameter of 52 mm were installed per bore hole (inside diameter, 22 cm); the piezometers usually had one screen above the leachate plume (Figure 1, positions a), one screen inside the leachate plume (positions b), and one screen below the leachate plume (positions c). The screens were 20 cm long. Samples from piezometer screens were designated by using the distance downstream of the landfill and the position of the screen; i.e., samples -200a and 0a were samples from screens above the leachate plume in a piezometer 200 m upstream and in a piezometer in the landfill (19 m from the downstream border), respectively.

Sampling. In September 1998, anaerobic groundwater samples were collected in sterile glass bottles by letting the bottles overflow, after 3 volumes of standing water in each piezometer was removed with a peristaltic pump. The bottles were capped with as small a headspace as possible. In October 1998, sediment cores were taken anaerobically with a core pushing device (Delft Geotechnics, Delft, The Netherlands) (7) at five locations (one upstream and four downstream) in the plume of leachate (Figure 1). After retrieval, the ends of the stainless steel cores (length, 20 cm; inside diameter, 30 mm) were immediately capped, and the cores were stored in a container, which was made anaerobic by flushing with nitrogen gas. Sediment cores and groundwater were transferred to the laboratory and stored for less than 24 h at 4°C. Next, 100 ml of groundwater was vacuum filtered with 45-mm-diameter, 0.2-μm-pore-size filters (Sartorius). Cores were sampled under a nitrogen atmosphere in an anaerobic glove box (Mecaplex). Several centimeters at the ends of the cores were not used. For molecular analysis, sediment and filters were frozen at -80°C until DNA isolation.

Chemical analysis. Oxygen content, pH, and electrical conductivity were measured in the field with electrodes placed in flow cells. Hydrochemical parameters (alkalinity; benzene, toluene, ethylbenzene, xylene, naphthalene, Mn, Fe, Si, Al, Mg, NH_4, Ca, K, Na, Cl, SO_4, H_2S, NO_2, NO_3, CH_4, and dissolved organic carbon contents) and sedimentological parameters (lime, humus, sand, clay, silt, carbon, and nitrogen contents) were determined by using Dutch NEN standards and laboratory procedures. Samples were grouped based on chemical characteristics by using principal-component analysis and cluster analysis (Systat 7).

DGGE profiling. DNA extraction was performed as described previously (40) A *Bacteria*-specific PCR was performed in a 25µl (total volume) mixture containing 0.4 µM primer F341-GC (35), 0.4 µM primer R518 (35), each deoxynucleoside triphosphate at a concentration of 0.4 mM, 10 µg of bovine serum albumin, Expand buffer (Boehringer, Mannheim, Germany), 2.6 U of Expand enzyme, and 1 µl of undiluted DNA template. Amplification was performed with a Perkin-Elmer DNA Thermo Cycler as follows: 94°C for 4 min, followed by 35 cycles of 94°C for 0.5 min, 54°C for 1 min, and 72°C for 1 min, and a final elongation at 72°C for 5 min. For profiling of *Archaea*, a nested approach was used. Primers pRA46f (37)and univ907r (6) were used to produce a 0.9-kb fragment, which after a 100-fold dilution was used as a template in an amplification reaction with primers pARCH340f and pARCH519r (37). Amplification was performed with the same settings as those used for *Bacteria*-specific amplification.

DGGE was performed with the Bio-Rad DCode system. The PCR product was loaded onto 1-mm-thick 8% (wt/vol) polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gels containing a 40 to 60% or 40 to 70% linear denaturing gradient for *Bacteria* and a 45 to 70% linear denaturing gradient for *Archaea*; 100% denaturant was defined as 7 M urea and 40% (vol/vol) formamide. The gels were electrophoresed in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM Na-EDTA; pH 8.0) at 70 V and 60°C for 16 h. The gels were stained in 1 TAE buffer containing 1 µg of ethidium bromide ml^{-1} and were recorded with a charge-coupled device camera system (The imager; Appligen, Illkirch, France). Gel images were converted, normalized, and analyzed with the GelCompar 4.0 software package (Applied Maths, Kortrijk, Belgium), using the Pearson product moment correlation coefficient and the unweighted pair group clustering method with arithmetic averages (UPGMA). To aid in conversion and normalization of gels, a marker consisting of 11 clones was added on the outsides of the gels, as well as after every four samples. The outer two lanes of each gel were not used. In all analyses the markers clustered over 95% similarity.

Cloning and sequencing of 16S rDNA. PCR primers 8f and 1512r (17) were used to amplify almost complete 16S ribosomal DNA (rDNA). Products cleaned with a Qiaquick

Rep purification kit [Qiagen, Hilden, Germany]) were cloned into *Escherichia coli* JM109 by using the pGEM-T vector system (Promega, Madison, Wis.). Transformants were checked for inserts of the correct size by performing a PCR with pGEM-T-specific primers T7 and Sp6. Products of the correct size were used as templates in a PCR with primers F341-GC and R518 to compare the band position in DGGE gels to that of the environmental sample from which the clone was derived. Sequencing PCR was carried out with an ABI PRISM dye terminator cycle sequencing core kit (Perkin-Elmer), and the purified products were electrophoresed on a SEQUAGEL-6 sequence gel (National Diagnostics, Atlanta, Ga.) with a 373A DNA sequencer (PE Biosystems, Applied Biosystems, Foster City, Calif.). At least the V3 region (*E. coli* positions 341 to 518) was sequenced, and a number of clones were sequenced completely. Both strands of the 16S rRNA gene were sequenced. Sequences were compared to sequences deposited in the GenBank DNA database by using the BLAST algorithm (5).

MPN-PCR. Serial twofold dilutions of DNA extracts were made in sterile water and used as templates for PCR. Most-probable-number PCR (MPN-PCR) of members of the family *Geobacteraceae* was performed with primers 8f and Geo825 (46). MPN-PCR numbers of members of the *Bacteria* were determined with primers 8f and R518. To account for variations in the efficiency of DNA extraction and recovery, the numbers of members of the *Geobacteraceae* were expressed relative to the numbers of members of the *Bacteria*.

Nucleotide sequence accession numbers. Nucleotide sequences have been deposited in the GenBank database under accession numbers AY013585 to AY013658 and AY013660 to AY013698.

RESULTS

Hydrogeochemistry of the plume of landfill leachate

Groundwater samples for hydrochemical and microbiological analyses were retrieved in September 1998 from 29 piezometers (Figure 1). An ordination plot constructed on the basis of the measured hydrogeochemical parameters (Figure 2) revealed clustering of the sampling points into three groups, two large clusters C and P1) and one small cluster (cluster P2). The two large clusters were mainly separated along the principal component 1 (PC1) axis, which explained 58.8% of the total variance. PC1 correlated strongly with the following parameters indicative of pollution by landfill leachate (correlation coefficients are given in parentheses): electrical conductivity (0.985), alkalinity (0.978), total inorganic carbon (0.977), magnesium (0.970), dissolved organic carbon (0.957), calcium (0.934), ammonium (0.929), potassium (0.894), chloride (0.891), and sodium (0.856). Cluster C (Figure 2) contained groundwater samples having low values for these parameters (slightly

polluted or clean), while clusters P1 and P2 contained samples that had high values for these parameters and therefore were obviously polluted. The grouping of the samples (Figure 2) corresponded exactly with the delineation of the plume by vertical continuous profiles of bulk conductivity obtained by cone penetration tests performed in May 1998 (48).

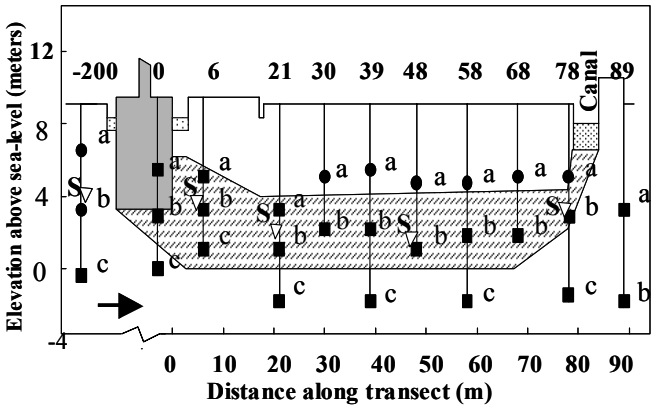

Figure. 1. *Cross section of Banisveld landfill (grey) and the plume of leachate (arced) downstream of the landfill, showing the locations of the 11 bore holes. Each bore hole is indicated by a number, corresponding to the distance from the downstream border of the landfill. 2 to 3 screens were placed per bore hole, indicated by a character (a, b or c) and a symbol: ●, screen from which in September 1998 a groundwater sample with a nitrate concentration >0.5 mg/l was withdrawn; ■, no nitrate present. ▽, sediment* (S) *sampled in October 1998.*

Clusters P1 and P2 were separated along the PC2 axis. This axis (which explains 16.3% of the variance) positively correlated with silica (0.860), ethylbenzene (0.781), xylene (0.759), and naphthalene (0.563) and correlated negatively with the reduced redox species Fe (II) (-0.733) and Mn(IV) (-0.617). Only cluster P2 samples (piezometer screens 0a and 0b) contained obvious concentrations of ethylbenzene (53 μg/liter) and xylene (120 μg/liter). These aromatic compounds were not present 6 m downstream of the landfill, while naphthalene had disappeared 21 m downstream. Benzene (maximum concentration, 28 μg/liter) was more persistent, and its concentration decreased along the flow path, to 6 μg/liter at 78 m from the landfill. The concentration of chloride (used as a conservative tracer, with a background concentration of 12 to 70 mg/liter upstream of the landfill) was constant (mean value in the plume of pollution, 270 mg/liter), indicating that the decreases in the concentrations of organic contaminants were not due to dilution. As the organic content of the sediment was low (<0.1%), sorption alone cannot explain the decreases (49).

Attenuation of organic contaminants in the plume appeared to occur under iron-reducing conditions. Oxygen (<0.1 mg/ liter) was not detected in any of the samples. Nitrate (>0.5 mg/ liter; maximum concentration, 76 mg/liter) was encountered only

upstream of the landfill and above the plume (Figure 1), indicating that denitrification is probably a dominant redox process at the top fringes of the plume. In the plume, Fe (II) concentrations in general increased along the transect, while the presence of a pool of Fe (III) oxyhydroxides and hydrogen concentrations (48) also indicated that iron reduction was a dominant redox process. Also, below the plume the absence of nitrate and the measured concentrations of hydrogen indicated that iron reduction was the dominant redox process.

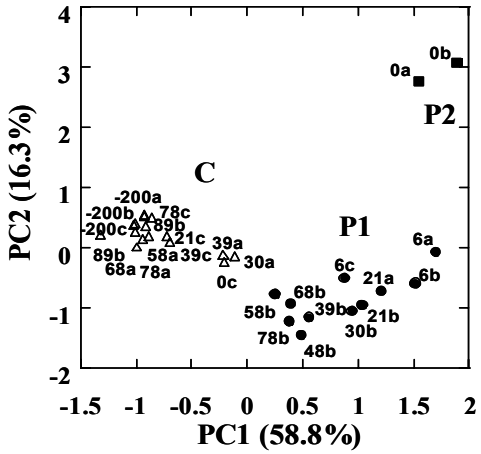

Figure 2. *Ordination plot produced from principal component analysis on hydrochemical parameters of groundwater samples from the aquifer surrounding Banisveld landfill. Three clusters of clean (C [△]) and polluted (P1[●] and P2[■]) groundwater samples are shown. The numbers and lowercase letters indicate the samples examined (see Figure 1).*

Microbial community structure of groundwater inside and outside the plume of leachate

Microbial communities in groundwater were profiled by DGGE of amplified 16S rDNA fragments. The profiles of the bacterial communities were complex, and the data revealed that there was a high degree of variation between samples (Figure 3A). To establish relationships between samples, the entire densitometric curves for the tracks were numerically compared by using the Pearson product moment correlation coefficient (40, 42). In general, cluster analysis with UPGMA grouped samples of polluted groundwater in one large cluster at a level of similarity of 35%, while clean samples clustered separately (Figure 3A). Only three DGGE profiles (those for samples 21c, 0c, and 78b) from the 29 groundwater samples examined did not cluster in accordance with the degree of pollution. There were clearly differences in microbial composition and thus community heterogeneity within the plume because samples from the plume clustered at a level of only 35%. Samples from within and just beneath the landfill (samples 0a and 0b; cluster P2 in Figure 2) and from 6 m downstream (samples 6a, 6b, and 6c; cluster P1 in Figure 2) produced the

most similar profiles. The bacterial communities in groundwater obtained from outside the plume showed more variation than those from within the plume. The nitrate-containing groundwater samples from above the plume (samples 30a, 39a, 48a, and 58a) clustered together, while samples from further downstream that also contained nitrate (samples 68a and 78a) clustered separately.

A more distinctive difference between community structures within and outside the plume was observed for archaeal communities (Figure 3B). The DGGE profiles were less complex than those observed for *Bacteria*. The profiles of samples from the plume contained a few strong dominant bands, resulting a strong correlation at >70% for most of the samples from the plume. Two of the dominant bands were clearly visible only in the profiles of polluted groundwater samples; interestingly, one of these bands was not present in the profiles of samples obtained furthest downstream from the landfill (samples 58b, 68b, and 78b). Archaeal PCR products were not obtained from any of the samples from below the plume.

Composition of microbial communities in groundwater

Analysis of clone libraries was used as a second method to characterize the microbial communities in groundwater, and this analysis allowed more detailed phylogenetic information on the microorganisms present in groundwater samples. It also generated more specific data on how community structure was affected by landfill leachate. The libraries were prepared from three groundwater samples, each representing one of the three clusters (Figure 2), and the samples were obtained from approximately the same depth, as follows: sample -200b from upstream (clean, cluster C), sample 0b from beneath the landfill (polluted, cluster P2), and sample 6b from downstream of the landfill (polluted, cluster P1).

Nearly complete 16S rDNA sequences of members of the *Bacteria* were amplified and cloned. Between 95 and 105 clones were screened per clone library. Clones, as well as the PCR fragments used for cloning, were reamplified with primers F341-GC and R518, and their DGGE profiles were compared to that of the original sample (Figure 4 and 5). The similarity between the results for directly amplified groundwater DNA samples and nested PCR data (amplification with the 1.5 kb PCR fragment used for cloning as template) was more than 80% (Figure 4). This indicates that the PCR required for cloning did not lead to an obvious cloning bias; the data for 74% (sample -200b) to 85% (sample 6b) of the clones matched bands in the community DGGE profiles.

Ninety-six clones were randomly selected, and the part of the cloned 16S rDNA that was also profiled by DGGE (corresponding to *E. coli* positions 341 to 518, including the V3 region) was sequenced. Later, 17 of the partially sequenced clones and seven additional clones, mainly clones with DGGE bands corresponding to dominant bands in the original profiles, were nearly completely sequenced. Sequencing nearly complete 16S rDNA did not result in assignment to phylogenetic groups that differed from those based on the V3 region. The majority of the clones resembled (facultatively) anaerobic and

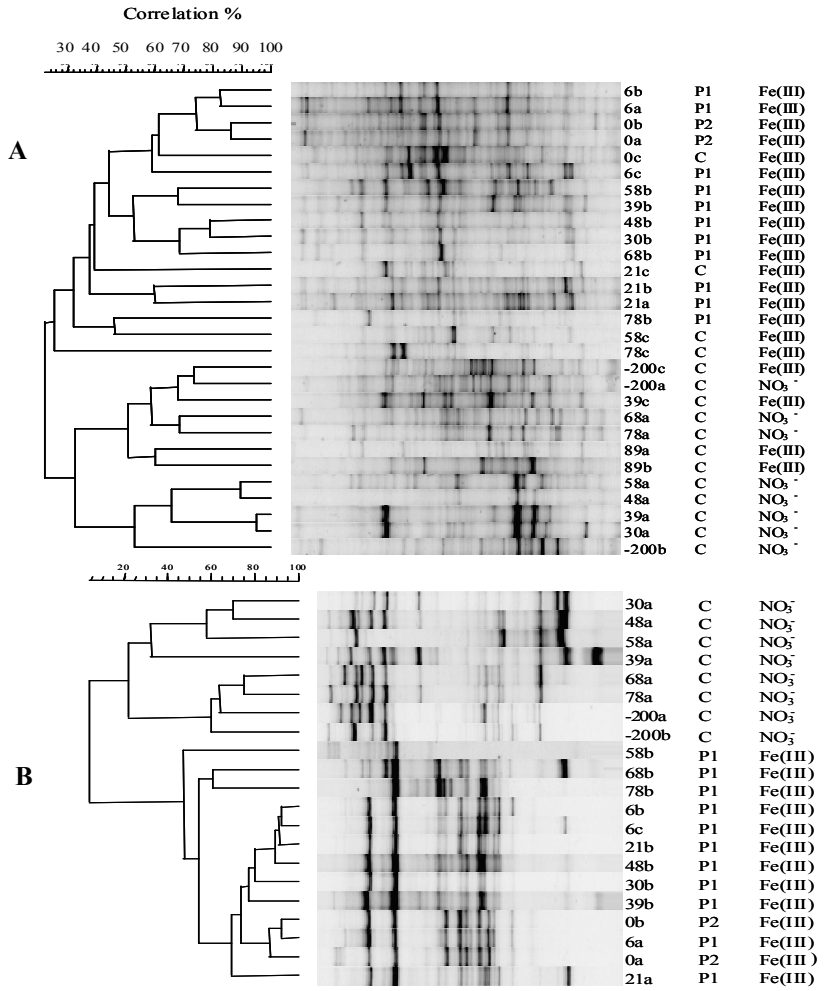

Figure 3. *UPGMA cluster analysis of DGGE profiles of Bacteria (**A**; 40-60% denaturant gradient) and Archaea (**B**; 45-70% denaturant gradient) in groundwater after Pearson product moment correlation. For each lane the sample designation (Figure 1), pollution level (P1, P2 and C refer to grouping in Figure 2), and proposed dominant redox process [NO_3^-, denitrification; Fe (III), iron-reduction] are indicated.*

microaerophilic microorganisms. Sequences related to facultatively anaerobic and microaerophilic microorganisms were especially observed with the upstream sample. No pathogens known were encountered. The distribution of the 96 randomly sequenced clones in phylogenetic groups is shown in Table 1; 16 to 25% of the sequences showed less than 90% similarity to sequences deposited in GenBank and were described as unclassified. It is obvious that the microbial composition of each groundwater sample was different. Upstream of the landfill there was strong dominance by bacteria belonging to the β subclass

of the class *Proteobacteria* (β-proteobacteria) (48.6%), which mainly resembled *Gallionella ferruginea* (four clones, 93 to 95% similarity) and *Azoarcus* sp. strain BS5.8 (five clones, 93 to 95% similarity). Linking of the clone identities to band positions in DGGE gels (Figure 5 and Table 2) indicated that these sequences also were related to dominant bands in the DGGE profile of the microbial community. Several sequences related to genera capable of denitrification (*Azoarcus*, members of the *Actinobacteria*) were found in this groundwater sample obtained from a denitrifying environment and also in the dominant bands (bands 2, 7, and 8 in Figure 5). Furthermore, two sequences related to sulfate reducers were encountered, and one of these sequences corresponded to a dominant band in the DGGE profile (band 9).

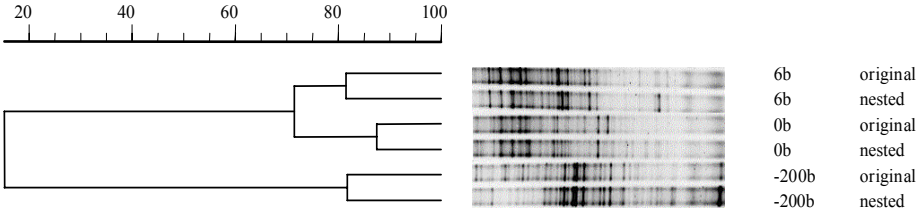

Figure. 4. *UPGMA cluster analysis of DGGE profiles (40-70% denaturant gradient) of groundwater samples – 200b, 0b and 6b used for constructing clone libraries. For each sample, the primers F341-GC and R518 were used directly on isolated groundwater DNA (original) or with the PCR fragment obtained with primers 8f and 1512r and used for cloning (nested).*

None of the clones from the groundwater beneath the landfill (sample 0b) showed affiliation to β-proteobacteria (Table 1). Here a strong dominance by Gram-positive bacteria was observed; 12.5% of the clones belonged to the high G+C content Gram-positive bacteria, and 37.5% belonged to the low G+C-content Gram-positive bacteria. The sequences of five clones (21%) closely resembled *Acetobacterium* sequences (95 to 98% similarity). These clones could be linked to dominant bands in the DGGE profile of the groundwater beneath the landfill (bands 10 and 11 in Figure 5). Another clone falling in the low G+C-content Gram-positive group also had mobility similar to that of a dominant band in the DGGE profile (band 13 in Figure 5), further demonstrating the apparent dominance of low G+C-content Gram-positive bacteria beneath the landfill. Only one sequence related to known iron reducers (*Geobacter*-like sequence) was encountered; this sequence was related to a subdominant band in the DGGE profile of the microbial community (band 12 in Figure 5).

Downstream of the landfill the relative number of low G+C content Gram-positive clones decreased, and β-proteobacteria reappeared (Table 1). The β-proteobacteria present were quite different from those encountered upstream of the landfill. Sequences related to *Acidovorax* (two clones, 93 to 96% similarity), *Rhodoferax*, and several uncultured β-proteobacteria were most frequently encountered in this clone library. Also, δ-proteobacteria, especially sequences related to the family *Geobacteraceae* (eight clones, 93

to 98% similarity), strongly contributed to the clone library (25.7% of the clones analyzed). Two clones, which based on sequencing of the V3 region were related to clone K20-06 (GenBank accession number AF145810), were also identified as *Geobacter spp*. Initially, four clones with similar migration in DGGE gels (band 16 in Figure 5) showed this affiliation after sequencing of the V3 region. Sequencing of nearly complete 16S rDNA of two of these clones showed that both were closely related to *Geobacter* sp. strain CdA2. Dominant bands in the DGGE profiles for groundwater samples obtained downstream of

Table 1. Relative levels of bacterial clones related to various phylogenetic groups in clone libraries from aquifer groundwater sample obtained upstream (sample -200b), beneath (0b) and downstream (6b) of Banisveld landfill.

Phylogenetic group	% in the following groundwater samples:		
	-200b[a]	0b[b]	6b[c]
Low-G+C Gram-positive group	2.9	37.5	11.4
High-G+C Gram-positive group	8.6	12.5	5.7
α-Proteobacteria	5.7	0.0	0.0
β-Proteobacteria	48.6	0.0	20.0
γ-Proteobacteria	0.0	8.3	0.0
δ-Proteobacteria	8.6	4.2	25.7
Green non-sulfur bacteria	2.9	4.2	5.7
Spirochaetales	0.0	8.3	0.0
Cytophaga-Flexibacter-Bacteroides	2.9	0.0	5.8
Holophaga	0.0	0.0	2.9
Verrucomicrobia	2.9	0.0	0.0
WS5 division	0.0	0.0	2.9
Unclassified (< 90% similarity)	16.9	25.0	20

a Principal-component analysis cluster C (Figure 2); the dominant redox process denitrification.
b Principal-component analysis cluster P2 (Figure 2); the dominant redox process iron reduction.
c Principal-component analysis cluster P1 (Figure 2); the dominant redox process iron reduction.

the plume also appeared to be contributed by members of the δ -proteobacteria (*Geobacteraceae*; bands 16 and 19 in Figure 5) and β-proteobacteria (bands 18 and 21 in Figure 5). The strong dominance by iron-reducing members of the *Geobacteraceae* is in agreement with iron reduction being the major redox process. One sequence related to a potential denitrifier (*Azoarcus* related) and another sequence related to a sulfate reducer were also encountered. The potential denitrifier showed comigration with five *Geobacter* clones (band 16) and corresponded to a dominant component of the DGGE profiles. As Figure 5 and Table 2 show, clones with different phylogenetic associations often exhibited similar migration patterns in DGGE gels.

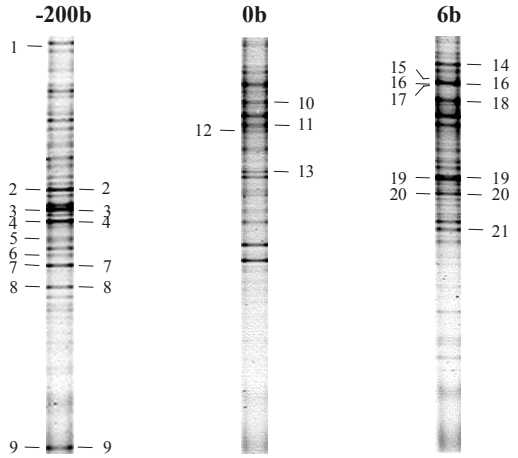

Figure 5. *Linking of bacterial clone identities to DGGE profiles (40 to 70% denaturant gradient) of groundwater samples taken upstream (sample -200b), beneath (sample 0b), and downstream (sample 6b) of Banisveld landfill. The band positions for clones that showed DGGE migration similar to that of a dominant band in the groundwater community DGGE profile are indicated to the right of each track. The band positions for clones with identities indicating an ability to perform redox reactions are shown to the left of each track. The identities of the numbered bands are given in Table 2.*

Confirmation that members of the *Geobacteraceae* were an important group of bacteria in the iron-reducing aquifer was obtained by an MPN-PCR analysis by using *Geobacteraceae* specific primers and expressing the number relative to the MPN obtained with general bacterial primers. Upstream the percentage was less then 0.5%, underneath the landfill the percentage was 6%, and downstream the percentage was 25%. Performing DGGE after a nested PCR with primers F341-GC and R518 on the *Geobacter*-specific PCR product revealed a dominant band corresponding to band 16 in Figure 5 for all iron-reducing samples (groundwater from the plume and below the plume). This band was not present in any of the denitrifying samples (data not shown).

As the clustering of DGGE profiles of *Archaea* appeared to be due to the presence or absence of two dominant bands (Figure 3B), only these bands were sequenced after excision from the gel. The sequence of the upper band was 100% similar to the sequence of methanogenic endosymbionts of the anaerobic protozoans *Trimyema compressa* (accession number Z16412) and *Metopus contortus* (accession number Z13957); the sequence of the lower dominant band was 96% similar to the sequence of an unidentified archeaon (accession number AF050617).

Geochemistry and microbial community structure of sediment.

In October 1998 sediment samples were retrieved from five locations, one upstream and four in the plume of leachate (Figure 1). Analysis of the chemical composition of the

sediment porewater and subsequent cluster analysis clearly revealed that the sediment samples from the plume were polluted and that the upstream sample was clean and did not cluster with the four sediment samples (data not shown). When parameters not affected by pollution (percentages of lime, humus, clay, silt, carbon, and nitrogen in sediment) were used for cluster analysis, a low-level relationship was observed (Figure 6A), indicating that the aquifer had a heterogeneous sediment composition. Sediment samples S[-200] and S[78] were most similar in terms of chemistry.

After numerical comparison of the DGGE profiles of *Bacteria*, the five sediment samples clustered together at the 60% level, and S[-200] and S[78] were most similar to each other (Figure 6B). Groundwater samples showed much less similarity. The profiles of sediment were quite different from the profiles of groundwater extracted from the same position and depth.

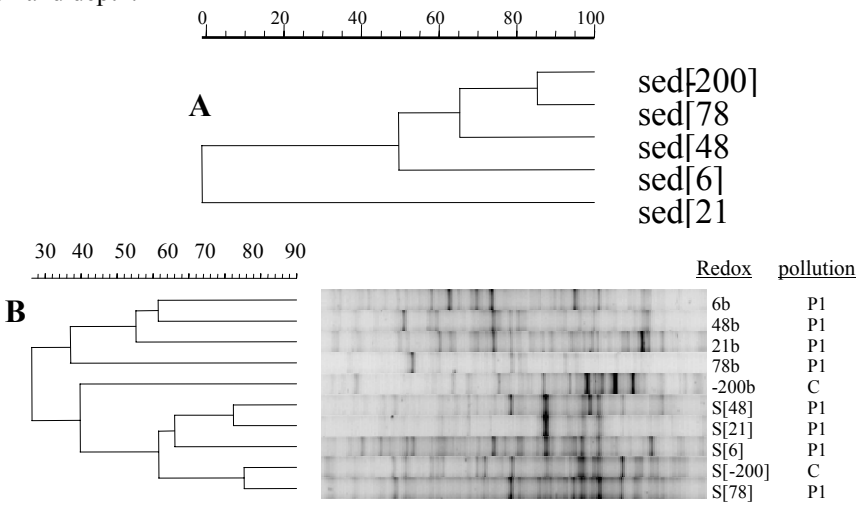

Figure 6. *UPGMA cluster analysis of pollution-independent sediment parameters (A) and Bacteria DGGE profiles of sediment and corresponding groundwater samples (40-60% denaturant gradient) (B). For each lane the sample designation (Figure 1; S[48], S[21], S[6], S[-200], and S[78] refer to sediment samples) and level of pollution (Figure 2) are indicated.*

DISCUSSION

In this study we attempted to relate microbial community structure to hydrochemistry in a landfill leachate-polluted aquifer. Microbial community structures were determined by cultivation-independent, molecular methods. The different steps DNA extraction, PCR, and profiling) in such a molecular approach have their pitfalls (50). However, since all samples were treated similarly, these pitfalls can be considered to be the same for all samples, allowing between-sample comparisons. The comparisons between samples were

Table 2. Identifies of clones related to numbered bands in Figure 5, as determined by partial or nearly complete 16S rDNA sequencing

Band	Accession no.	Closest relative in GenBank (accession number)	Similarity%	Phylogenetic group
1	AY013676[a]	*Desulfosporosinus* sp. *S10* (AF07527)	96	Low G+C gram positive
2	AY013696[a,b]	*Gallionella ferruginea* (L07897)	94	β-Proteobacteria
	AY013693	Uncultured Duganella CTHB-18 (AF067655)	93	β-Proteobacteria
	AY013698 [a,b]	*Gallionella ferruginea* (L07897)	93	β-Proteobacteria
	AY013694 [a,b]	*Gallionella ferruginea* (L07897)	95	β-Proteobacteria
	AY013691[a]	*Actinomyces* sp. (X92701)	96	High G+C gram positive
	AY013663	Uncultured bacterium RB25 (Z95718)	88	Unclassified
3	AY013670	Unidentified beta proteobacterium cda-1 (Y17060)	96	β-Proteobacteria
	AY013688	Unidentified beta proteobacterium cda-1 (Y17060)	96	β-Proteobacteria
4	AY013697 [a,b]	*Gallionella ferruginea* (L07897)	92	β-Proteobacteria
	AY013695[b]	Unidentified bacterium BD4-9 (AB015559)	88	Unclassified
5	AY013690[a]	*Azoarcus* sp. BS5-8 (AF011350)	93	β-Proteobacteria
6	AY013666[a]	*Azoarcus* sp. BS5-8 (AF011350)	93	β-Proteobacteria
7	AY013669[a]	*Azoarcus* sp. BS5-8 (AF011350)	94	β-Proteobacteria
	AY013681[a]	*Azoarcus* sp. BS5-8 (AF011350)	94	β-Proteobacteria
	AY013675[a]	*Azoarcus* sp. BS5-8 (AF011350)	93	β-Proteobacteria
	AY013664[a]	*Denitrifying bacterium* 72Chol (Y09967)	95	β-Proteobacteria
	AY013682	*Candidate division* OP11 clone OPd29 (AF047561)	90	Unclassified
	AY013689	Uncultured bacterium t0.6.f (AF005745)	91	Green non-sulfur bacteria
8	AY013674	Unidentified bacterium DGGE band 10 (AJ009652)	98	High G+C gram positive
	AY013684[a]	*Azoarcus* sp. BS5-8 (AF011350)	98	β-Proteobacteria
9	AY013665[a]	*Desulfovibrio aminophilus* (AF067964)	93	δ-Proteobacteria
	AY013607[b]	*Acetobacterium malicum* (X96957)	95	Low G+C gram positive
	AY013610[b]	*Acetobacterium malicum* (X96957)	97	Low G+C gram positive
10	AY013593	*Acetobacterium carbonolicum* (X96956)	98	Low G+C gram positive
	AY013613	*Acetobacterium wieringae* (X96955)	97	Low G+C gram positive
11	AY013591	*Acetobacterium wieringae* (X96955)	98	Low G+C gram positive
12	AY013609 [a,b]	*Geobacter akaganeitreducens* (U96918)	94	δ-Proteobacteria
13	AF013603	Uncultured eubacterium WCHB1-21 (AF505080)	96	Low G+C gram positive
14	AY013658	Uncultured freshwater bacterium (AF109142)	98	Unclassified
15	AY013644 [a,b]	*Geobacter* sp. *CdA-2* (Y19190)	96	δ-Proteobacteria
	AY013648 [a,b]	*Geobacter* sp. *CdA-2* (Y19190)	94	δ-Proteobacteria
	AY013647 [a,b]	*Geobacter* sp. *CdA-2* (Y19190)	96	δ-Proteobacteria
	AY013651[a]	*Geobacter* sp. *CdA-3* (Y13131)	93	δ-Proteobacteria
	AY013642[a]	Metal contaminated soil clone K20-06(AF145810)	95	δ-Proteobacteria
16	AY013634[a]	Metal contaminated soil clone K20-06(AF145810)	98	δ-Proteobacteria
	AY013641[a]	*Azoarcus sp. PCR strain* (X85434)	93	β-Proteobacteria
	AY013649[b]	Uncultured bacterium WCHB1-60 (AF050598)	91	Candidate division WS5
17	AY013652 /3[a,c]	*Geobacter* sp. (GSPY19190)	96	δ-Proteobacteria
	AY013650[b]	*Acidovorax devluvii* (Y18616)	91	β-Proteobacteria
18	AY013646[b]	*Acidovorax* sp. UFZ-B517 (AF235010)	96	β-Proteobacteria
	AY013643[b]	*Rhodoferax fermentas* (D16211)	96	β-Proteobacteria
19	AY013645 [a,b]	*Geobacter sp.* CdA-2 (Y19190)	95	δ-Proteobacteria
	AY013633	*Eubacterium limosum* (M59120)	94	Low G+C gram positive
	AY013638	Uncultured bacterium clone H1.4.f (AF005748)	93	Green non-sulfur bacteria
20	AY013620[a]	Uncultured sulfate-reducing bacterium 368 (AJ389629)	91	δ-Proteobacteria
21	AY013625	Uncultured clone CRE-FL35 (AF141457)	97	β-Proteobacteria

a. The identity of the closest relative in the GenBank database gives an indication of the ability to perform redox reactions (microaerophilic, denitrification, iron eduction, or sulfate reduction).

b. The 16S rDNA was almost completely sequenced.

c. E. coli positions 8 to 518 and 1002 to 1512 were sequenced.

accomplished by numerical analysis of DGGE profiles, using the Pearson product moment correlation coefficient. This coefficient is robust and objective, since whole curves are compared and subjective band scoring is omitted (40). Difficulties with band assignment are especially likely to occur with highly complex and varying profiles, as in our study. Furthermore, the Pearson coefficient does not suffer from mismatches between peaks and shoulders, a problem often found when band scoring is used (40), and is much less laborious.

Comparison between microbial community structures of groundwater and sediment. In contrast to the groundwater results, no relationship to pollution was apparent from the analysis of the microbial community structure of sediment. The number of particle-bound microorganisms per gram of sediment is usually 1 order of magnitude higher than the number of free-living microorganisms per milliliter in landfill leachate polluted aquifers (4, 22). Since 1 cm3 of sediment weights 2.65 g and contains about 30% water, the number of sediment associated microorganisms is about 2 orders of magnitude higher than the number present in water. Given that on a geological scale a relatively short time has elapsed since landfilling started (1965), leachate may have had little impact on the microorganisms closely associated with the 10,000 to 100,000 year-old sediments. A large portion of the sediment bound microorganisms could be physically (i.e., in pores) or biologically (i.e., inbiofilms) protected from the influence of leachate. Furthermore, the pollutant-independent heterogeneity of sediment composition (Figure 6A) may have contributed to variability in microbial community structure (33) and hampered observation of changes related to pollution. The differences in community structure between sediment and nearby groundwater are in agreement with previous observations made at landfill leachate-polluted aquifers (42) and other environments for which communities of particle-bound and free living bacteria were determined (15, 24).

Groundwater community structure in relation to pollution and redox processes
In the leachate plume examined in this study, iron reduction is a dominant redox process, and in the zone of iron reduction BTEX compounds appear to be degraded. Similar observations have been made for other landfill leachate-affected aquifers (2, 21, 34, 44). Both DGGE and clone library data indicate that the microbial community structure of the iron-reducing leachate plume differs considerably from the microbial community structure of the unpolluted groundwater upstream, above, and below the plume of pollution. Clustering of DGGE profiles of *Bacteria* showed that 90% of the samples were correctly separated based on the level of pollution. Two clean samples (samples 0c and 21c) were identified as polluted, and one polluted sample (sample 78b) was identified as clean. The latter sample was from the piezometer in the plume that was farthest from the landfill and thus was influenced by landfill leachate for the shortest time. The values for some

hydrochemical parameters of sample 0c, such as chloride concentration, were remarkably high for a clean sample (data not shown). Sample 21c was also the only sample wrongly assigned when culture-dependent anaerobic community-level physiological profiling was used (41). All DGGE profiles of *Archaea* were assigned to the correct cluster, based on the level of pollution. Thus, groundwater sampling was shown to be suitable for determining differences in microbial community structure associated with pollution. Microbial degradation can also be determined by using only groundwater samples, although the degradation rates are lower and groundwater sometimes exhibits lower degradation potential than aquifer sediment (3, 22).

Analysis of DGGE profiles showed that while communities of *Archaea* and *Bacteria* in the plume clustered together, more variation was observed outside the plume. Outside the plume more variation in dominant redox processes was found; denitrification occurred upstream and above the plume, and iron reduction occurred below the plume. Clustering of DGGE profiles of *Bacteria* correlated partially with these differences in redox processes. Communities of *Archaea* were clearly different, in the sense that all samples from iron-reducing, nonplume locations failed to yield a PCR product in the *Archaea*-specific PCR, while samples from locations characterized by denitrification did give rise to a PCR product. Cluster analysis of DGGE profiles of the latter samples showed that the profiles grouped together and were different from those of the communities of *Archaea* in the leachate plume.

The results for the clone libraries linking particular organisms to bands in DGGE profiles were consistent with the observed redox conditions. Upstream, where denitrifiying conditions prevailed, sequences related to potential denitrifiers (*Azoarcus* (52), members of the *Actinobacteria* (45)), as well as the microaerophilic iron-oxidizing organism *G. ferruginea* (19), were encountered. Sequences related to aerobic and denitrifying bacteria were seldom encountered beneath and downstream of the landfill. Beneath the landfill strictly anaerobic, fermentative microorganisms, especially members of the *Clostridiaceae*, dominated. Also, one sequence related to the *Geobacteraceae* was encountered. Downstream, where iron-reducing conditions dominated, a high percentage of the sequences (22%) was closely related to this family. Iron reduction is a general trait of cultivated members of the *Geobacteraceae* (26). Downstream one sequence related to a potential denitrifier (*Azoarcus*) and one sequence related to a sulfate reducer were obtained, while upstream two sequences related to sulfate reducers were also obtained. Culture-dependent studies of a Danish landfill leachate plume also showed that usually several types of redox reaction-performing microorganisms are present at the same location, even when redox conditions are unfavorable (33). The occurrence of specific phospholipid fatty acid (PLFA) biomarkers paralleled the occurrence of sulfate and iron reduction in the Danish aquifer (33).

Community structure and degradation in the leachate plume

While cluster analysis of DGGE profiles obtained with general bacterial and archaeal primers was able to separate communities from polluted groundwater and clean groundwater, it was not able to clearly distinguish samples within the plume and to relate them to hydrochemistry or processes. In part, this might have been due to the fact that iron reduction is the dominant redox process throughout the plume. Clustering of the DGGE profiles of members of the *Bacteria* revealed separation of samples close to the landfill (sampling wells 0 and 6) from samples farther away, but based on hydrochemistry the samples obtained near the landfill were members of cluster P1 (hardly any BTEX compounds) and P2 (containing BTEX compounds) (Figure 2) and thus could not be clearly related to degradation. The lack of a relationship between microbial community structure and degradation is not surprising since (i) xenobiotic compounds (primarily BTEX [<204 μg/liter]) contribute less than 1% of the dissolved organic carbon (57 to 98 mg/liter) in the plume and thus microorganisms metabolizing BTEX make only a minor contribution to the total microbial community and (ii) in addition to organic carbon, microorganisms leach from the landfill and strongly contribute to the rDNA-based microbial community structure, although they are not active. Leaching of *Bacteria* is indicated by the fact that the DGGE profile of the groundwater sample from just below the landfill (sample 0b) is very similar to the DGGE profile of the sample taken from within the landfill (sample 0a). Also, the clone libraries from groundwater beneath and downstream of the landfill revealed a large number of sequences related to complex-compound-degrading fermentative bacteria and acetogens (the genera *Acetobacterium*, *Clostridium*, *Cytophaga*, *Spirochaeta*, and *Bacteroides*). In landfills, high numbers (>10^7 cells per g [dry weight]) of acetogenic, xylanolytic, and cellulolytic bacteria are present, while only simple organic compounds leach out (7). A large number of *Clostridium*- and *Cytophaga*-like sequences were also detected in a molecular study of a Canadian landfill (25).

Microorganisms can persist in groundwater over long distances; anaerobic microorganisms from livestock wastewater constituted a major part of the microbial community at an aerobic sampling well 400 m from the point of pollution (9). Although molecular analysis of rRNA instead of rDNA is thought to be more useful as it should favor the detection of the active microbial community (17), it is unlikely to be of much benefit for studying environments such as those examined in this study. Starved bacteria can maintain high numbers of ribosomes, up to 30% of the maximum (18). Furthermore, if one assumes that indeed there is a universal relationship between RNA/DNA ratio and growth rate (μ) and that this relationship can be described by $\text{RNA/DNA} = 1.65 + 6.01\ \mu^{0.73}$ (23), then even if microorganisms were growing in their natural environment at the unrealistically high rate of 0.5 h^{-1} (generation time, 80 min), their RNA/DNA ratio (with the RNA mainly being rRNA) is only three times higher than the ratio under zero-growth conditions. In the

subsurface, growth rates can be assumed to be much lower (51). Therefore, like rDNA-based analysis, rRNA-based analysis indicates merely presence and not activity.

High methane concentrations in the groundwater indicated that there were methanogenic conditions in the landfill; thus, leaching of archaeal cells from the landfill might be expected. Remarkably, one of the dominant bands in the archaeal profiles was clearly related to a methanogenic endosymbiont of an anaerobic protozoan. This suggests the presence of anaerobic protozoans. Pollution usually increases protozoan numbers (36), although no protozoans could be detected in a Danish landfill leachate-polluted aquifer (33). Predation by protozoans and variations in hydrochemical composition in the plume could explain why despite the clustering considerable variation (profiles clustered only at the 35% level) was found in microbial community structure in the leachate plume.

Multivariate analysis of the relationship between PLFA profiles and microbial redox processes revealed that PLFA profiles also had limited value for identifying more specific microbial communities in a landfill leachate plume (32). It is well known that some numerically minor groups of microorganisms are essential for major environmental processes; i.e., nitrifiers are essential in the N cycle (38). In contrast to PLFA, specific functional groups of microorganisms can be more adequately investigated by molecular methods, such as those used in this study. Our limited knowledge concerning genes involved in anaerobic BTEX degradation (20) eliminates any possibility of direct measurement of degradation-related gene expression. However, molecular techniques linking community structure to function have recently been developed. Use of stable-isotope probing (39) or bromodeoxyuridine labeling (47)in carefully designed microcosm assays that mimic the natural situation as closely as possible should help establish a clearer relationship between microbial community structure and degradation processes. Also, for this aquifer, in which iron reduction is a major redox process and degradation occurs under these redox conditions, a logical choice for future research is to focus on iron-reducing bacteria. While iron-reducing bacteria are phylogenetically very diverse (8, 13, 16, 26, 28), only sequences related to the *Geobacteraceae* were encountered. Clone libraries linking identities to DGGE profiles of whole microbial communities and MPN-PCR revealed the considerable contribution of *Geobacteraceae* to the microbial community. The results presented here underline the finding that members of the *Geobacteraceae* are widely distributed and dominant in diverse iron-reducing environments (14, 46). Interestingly, until now only members of the genus *Geobacter* have been found to be capable of toluene oxidation under iron reducing conditions (14, 30) while there are strong indications that members of the *Geobacteraceae* are also involved in anaerobic benzene degradation (43). Members of the *Geobacteraceae* are also important humic acid reducers (12) and are capable of using humic acids as electron shuttles to facilitate iron reduction (31). Humic acids account for about 10% of the dissolved organic carbon in landfill leachate (10). Consequently, members of the *Geobacteraceae* are a good first choice for more detailed community studies in relation to natural attenuation in landfill leachate-polluted aquifers.

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Chapter 3

Geobacteraceae community composition relates to hydrochemistry and biodegradation in an iron-reducing aquifer polluted by a neighboring landfill

ABSTRACT

Relationships between community composition of the iron-reducing *Geobacteraceae*, pollution levels and occurrence of biodegradation were established for an iron-reducing aquifer polluted with landfill leachate, using cultivation-independent *Geobacteraceae* 16S rDNA-targeting techniques. Numerical analysis of denaturing gradient gel electrophoresis (DGGE) profiles, and sequencing, revealed a high *Geobacteraceae* diversity and showed that community composition within the leachate plume differed considerable from the unpolluted aquifer. This suggests that pollution has selected for specific species out of a large pool of *Geobacteraceae*. DGGE profiles of polluted groundwater taken near the landfill (6-39 m distance) clustered together. DGGE profiles from less-polluted groundwater taken further downstream did not fall in the same cluster. Several individual DGGE bands were indicative of either the redox process or the level of pollution. This included a pollution-indicative band that dominated the DGGE profiles from groundwater samples taken close to the landfill (6-39 m distance). The clustering of these profiles and the dominance by a single DGGE band corresponded to the part of the aquifer where organic micropollutants and reactive dissolved organic matter were attenuated at relatively high rates.

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INTRODUCTION

Iron is one of the most abundant elements in the earth crust and in many subsurface environments its reduction is the predominant microbial redox process (16). Because the degradation of organic compounds leads to the rapid depletion of oxygen and nitrate, iron reduction frequently becomes dominant after pollution with organic matter (3, 16).

Iron reduction is also the major redox process in many landfill leachate-polluted aquifers (4, 30). In the past, landfills were not lined and leachate could contaminate aquifers with a complex mixture of organic and inorganic compounds. Natural attenuation of organic compounds in leachate-polluted groundwater, including those of toxic aromatic compounds like toluene and benzene, is especially observed under iron-reducing conditions (4, 30).

Natural attenuation under iron-reducing conditions also occurs in the aquifer underlying the Banisveld landfill, The Netherlands (Chapter 2, 22, 30). Molecular fingerprints of *Bacteria* and *Archaea* communities have been found to be related to the presence of pollution and the type of redox process at this location, but no such correlations were observed with biodegradation of dissolved organic carbon (DOC) or BTEXN (benzene, toluene, ethylbenzene, xylene and naphthalene) (Chapter 2, 22). Members of the family *Geobacteraceae* accounted for a considerable proportion of the microbial community in the polluted aquifer, up to 25% of bacterial counts (Chapter 2, 22). Enrichment of *Geobacteraceae* generally occurs upon the stimulation of dissimilatory metal reduction by the introduction of organic electron donors into aquifer sediments (2, 10, 11, 20, 25). *Geobacter metallireducens* and *G. grbiciae* are the only iron-reducing species described to date that are capable of aromatic hydrocarbon degradation (5, 14), while *Geobacter* spp. were implicated in anaerobic benzene degradation (23). Therefore, we proposed that *Geobacteraceae* are responsible for much of the biodegradation of organic compounds in landfill leachate (Chapter 2, 22). If so, more detailed knowledge on the diversity and community structure of *Geobacteraceae* should improve insight into the link between microbial community composition and natural attenuation of landfill leachate. This knowledge will aid in the development of monitoring and bioremediation strategies.

Here, we report the results of cultivation-independent, *Geobacteraceae*-specific molecular analyses on groundwater samples from the aquifer underlying the Banisveld landfill. The community composition and diversity of *Geobacteraceae* are indeed related to the occurrence of degradation processes in, and the hydrochemistry of, the polluted aquifer.

MATERIALS AND METHODS

Site description. The Banisveld landfill is located 5 km southwest of Boxtel, The Netherlands. Household refuse and illegal waste were discarded in a 6 m deep sand pit between 1965 and 1977. In June 1998, a transect of 11 bailer drillings was installed, along

the direction of groundwater flow (Figure1). Each borehole had two or three PVC piezometers, usually one screen above (numbered 'a' in Figure 1), one inside (b) and one below (c) the leachate plume. Samples from piezometer screens were designated by using the distance downstream of the landfill and the position of the screen, i.e. sample 39b is a sample from the plume, 39 m downstream. Extensive hydrochemical characterization was performed in 1998 and 1999 (30). Within the plume, concentrations of dissolved organic matter, naphthalene (N) and the aromatic micropollutants benzene (B), ethylbenzene (E) and xylene (X) decrease, with naphthalene, ethylbenzene and xylene disappearing within the first 21 m (30). Other micropollutants, such as chlorinated hydrocarbons, were never detected (GC-FID/MS detection limit of 0.2 µg/l). The micropollutants (max. 221 µg/l) formed a small fraction of the dissolved organic carbon (DOC; 62-110 mg/l) in the plume of pollution (30). Reactive transport modeling indicated that DOC consisted of a persistent (67% of DOC) and a reactive (33%) fraction underneath the plume (29). The reactive fraction was degraded with a first order rate constant of 1.06 10^{-1} yr^{-1} and was nearly completely consumed in the first 39 m downstream of the landfill. The persistent fraction was degraded much slower, with a first order rate constant of 1.03 10^{-2} yr^{-1}.

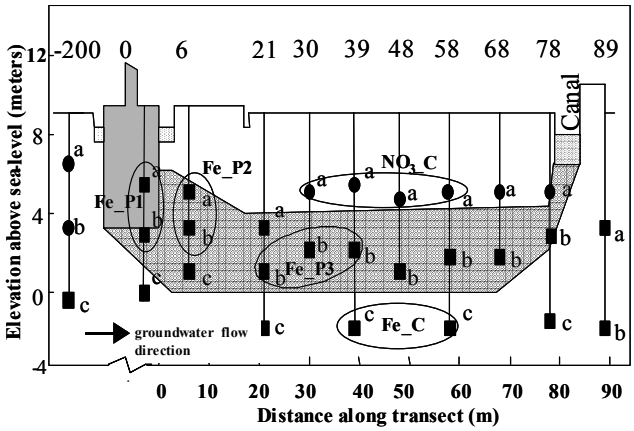

Figure 1. *Cross section of Banisveld landfill (shaded area) and the plume of leachate (cross-arched area) downstream of the landfill, demonstrating the locations of the 11 bore holes. Each borehole is indicated by a number corresponding to the distance (in meters) from the downstream border of the landfill. 2 to 3 screens were placed per borehole, indicated by a character (a, b or c) and a symbol: ●, screen from which in September 1998 a groundwater sample with a nitrate concentration >0.5 mg/l was withdrawn; ■, no nitrate present. The five oval circles and their codes refer to Table 1. DNA extracts from groundwater samples taken from screens within the oval circles were pooled and used for the construction of Geobacteraceae clone libraries. Additional characteristics of the research site are given in Material and Methods.*

Only 10% of the persistent DOC was degraded in the first 39 meters downstream of the landfill. As inferred from a combination of hydrogen-gas measurements, analysis of redox species (oxygen, nitrate, Fe (II), sulfide, sulfate, methane), thermodynamic calculations and

inverse geochemical modeling, iron reduction was the dominant redox process inside and beneath the plume, while nitrate reduction was observed above the plume (see Figure 1) (30). Nitrate reduction above the plume was also indicated by both an enriched $\delta^{15}N\text{-}NO_3$ and partial N_2 pressure exceeding atmospheric equilibrium (30). The maximum rate of iron-reduction determined by reactive transport modeling was 1.5 10^{-3} mol/liter/year below the landfill and decreased with distance from the landfill (29).

***Geobacteraceae*-specific DGGE profiling and data analysis.** The same DNA extracts previously used to profile *Bacteria* and *Archaea* communities, were used in this study (Chapter 2, 22). These DNA extracts were isolated from groundwater sampled in September 1998 and stored at –20°C for 3 years before starting the work described in this paper.

To profile *Geobacteraceae* communities in DGGE, a nested PCR approach was applied. First, a *Geobacteraceae*-specific PCR (25) was performed to amplify a 0.8 kb 16S rRNA gene fragment in a total volume of 25 µl, containing 0.4 µM primer 8f (8), 0.4 µM primer 825r (25), 0.4 mM dNTPs, 10 µg BSA(Biolabs, UK), 'Expand buffer' and 2.6 U 'Expand enzyme' (Boehringer, Mannheim, Germany) and 1 µl of undiluted DNA extract. PCR was performed in a Perkin Elmer DNA Thermo Cycler as follows: 94°C for 4 min, then touch-town primer annealing from 65°C to 56°C (decreasing 1°C per 2 cycles), followed by 15 cycles at 55°C annealing temperature, with a final elongation at 72°C for 5 min. PCR products were purified (Qiaquick Rep purification kit, Qiagen) and 1 µl of 1/100 diluted PCR product was used for the second round of amplification using *Bacteria*-specific primers, in a 25 µl reaction volume containing 0.4 µM primer F357-GC (19), 0.4 µM primer R518 (19), 0.4µM dNTPs, 10 µg BSA, and 2.5U Taq polymerase. Amplification was performed as follows: 94 ^{0}C for 4 min, after which 35 cycles of 94 ^{0}C for 1 min, 54 ^{0}C for 1 min and 72 ^{0}C for 1 min, with a final elongation phase at 72 ^{0}C for 5 min.

DGGE was performed with the Bio-Rad DCode^{TM} system. PCR product was loaded onto 1 mm thick 8% (wt/vol) polyacrylamide (37.5:1 acrylamide:bisacrylamide) gels containing a 30-55% linear denaturing gradient. One hundred percent denaturant is defined as 7 M urea and 40% (v/v) formamide. Gels were run in 1 x TAE buffer (40 mM Tris-acetate, 1 mM Na-EDTA, pH 8.0) at 200 V for 4 hours. Gels were stained in 1 x TAE buffer containing 1 µg ml^{-1} ethidium bromide and recorded with a CCD camera system (The imager, Appligen, Illkirch, France).

Gel-images were converted, normalized and analyzed by the GelCompar II software package (Applied Maths, Kortrijk, Belgium). To facilitate the conversion and normalization of gel images, a marker consisting of 12 clones was added. DGGE profiles were compared using a band assignment-independent method (Pearson product-moment correlation coefficient and unweighted pair-group clustering method using arithmetic averages (UPGMA)), as well as a method based on band presence/absence (Jaccard

coefficient; $S_J = \frac{n_{AB}}{n_A + n_B - n_{AB}}$, in which n_A, n_B and n_{AB} are respectively the total number of bands in track A, in track B and the number of bands common to track A and B). The Pearson product moment correlation coefficient analysis is affected much less than band-based similarity coefficients by the amount of PCR products loaded on gel and is a fast, objective method to compare microbial community profiles (32). In band-assignment, a 1% band position tolerance (relative to total length of the gel) was applied, which indicates the maximal shift allowed for two bands in different DGGE tracks to be considered as identical. Band presence or absence in DGGE tracks was scored as 1 or 0 respectively. These 1/0 numbers were exported to the spreadsheet program Excel. To determine whether a certain DGGE band was specific for a certain condition, statistical analyses on 2 x 2 tables, containing the number of times the particular band was absent or present for a certain condition (clean vs. polluted, nitrate- vs. iron-reducing), were conducted by Fisher's exact test using Systat 7.0 (SPSS Inc).

Phylogenetic analysis of *Geobacteraceae* 16S rRNA genes. Five clone libraries were constructed. Each library corresponded to a combination of a particular pollution level (P, polluted; C, clean) and redox process (NO_3, nitrate- reducing; Fe, iron reducing) and were coded Fe_P1, Fe_P2, Fe_P3, Fe_C and NO_3_C, (see Figure1, Table 1). The clone libraries were constructed from composite samples, obtained by mixing equal amounts of isolated DNA from the relevant groundwater samples (see Table 1). A *Geobacteraceae*-specific PCR with primers 8f and 825r was performed as described above. PCR products were cleaned with the Qiaquick Rep Purification Kit (Qiagen, Germany), and cloned into *Escherichia coli* JM109 via the pGEM-T vector system (Promega, Madison, Wis., USA). Clones were screened by PCR with pGEM-T-specific primers T7 and Sp6. PCR products from transformants with correctly sized insert DNA were used as template in a PCR with *Bacteria*-specific primers F357-GC and R518 to compare the migration position in DGGE to the DGGE pattern of the environmental sample from which the clone had been derived: Clones were classified into DGGE types based on differences in migration behavior in DGGE. At least one representative clone per type was sequenced. Sequencing PCR was carried out with the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Core Kit (Perkin Elmer) and the purified products were run on a SEQUAGEL-6 sequence gel (National Diagnostics, USA) in a 373A/DNA Sequencer (Applied Biosystem, USA). Both strands of the 16S rRNA gene were sequenced, from *E. coli* position 8 to 825. Sequences were compared to sequences deposited in GenBank DNA database by using the BLAST algorithm to obtain the most closely related sequences (1). Putative chimera checks of the 16S rRNA gene sequences of clones were performed via the Chimera-Check-program from RDP (18) and by comparing phylogenetic trees based on the first 400 bp to those based on *E.coli* positions 401 to 825. Chimeric sequences were excluded from further phylogenetic

analysis. Sequence alignment was performed by clustalW and then corrected manually. Distance analysis on unambiguously aligned sequences using the correction of Jukes and Cantor (12) and bootstrap resampling (100 times) the TREECON package (31) and the distance matrix was used to construct a tree via the neighbor-joining method (24).

Molecular detection of *Anaeromyxobacter*, *Geothrix* and *Shewanella*. *Geothrix* and *Shewanella*-specific PCR were carried out as described by Snoeyenbos-West et al (25). *Anaeromyxobacter*-specific PCR was performed according to North et al. (20).

Nucleotide sequence accession number. Nucleotide sequences have been deposited in the GenBank database under accession number AY752746 to AY752785.

RESULTS

Molecular detection of specific groups of iron reducers

Iron reducing microorganisms from the genera of *Shewanella, Geothrix, Anaeromyxobacter* and *Geobacter* are common to various metal-reducing environments. Groundwater samples from the aquifer near the Banisveld landfill were tested for the presence of these microorganisms, using group-specific PCR amplification. Hydrochemical characteristics (redox conditions, presence of organic pollutants) of these groundwater-samples have been described previously (Chapter 2, 22, 29, 30) and are indicated in Figure 1 as well as shortly described under the heading 'site description' in Material and Methods. *Shewanella* was not detected in any of the composite DNA samples used to generate clone libraries (Table 1), despite the ability of the PCR assay to detect one 16S rRNA gene per amplification reaction (data not shown). After *Geothrix*-specific amplification, low-intensity PCR bands were observed, but only for the composite samples Fe_P2, Fe_P3 and NO_3_C. *Anaeromyxobacter* sequences were only detected in composite sample Fe_P1. By contrast, *Geobacteraceae*-specific PCR gave a strong signal for the 5 composite samples, as well as for all 27 individual groundwater sampling locations, indicating that *Geobacteraceae* are widespread in the aquifer. Combined with previous results indicating the dominant contribution of *Geobacter* spp. to microbial communities in the iron-reducing leachate plume (Chapter 2, 22), the results of these molecular analyses warranted further focus on iron-reducing *Geobacteraceae.*

Geobacteraceae community profiling

Geobacteraceae communities in groundwater were investigated by DGGE profiling of *Geobacteraceae*-specific 16S rRNA genes. A large diversity was observed (Figure 2). In total, sixty-two different banding positions were detected for the 27 groundwater samples analyzed. The average number of banding positions per groundwater sample was 16 with a minimum of 10 for sample 21c and a maximum of 24 for groundwater sample -200a. There

was no significant difference in average number of bands between groundwater samples stemming from the polluted and groundwater samples coming from the unpolluted part of the aquifer (ANOVA, $p>0.05$).

In order to detect similarities between DGGE fingerprints and to relate these to hydrochemical characteristics, cluster analysis was performed. The analysis was based on the whole densitometric curve of the DGGE profiles and used the Pearson product-moment correlation coefficient (21). Overall, a low similarity was found between samples in terms of their *Geobacteraceae* community profiles, often also when samples from locations of similar redox conditions and pollution levels were compared (Figure 2). Only samples from the iron-reducing, polluted part close to the landfill (at a distance of 6–39 m downstream of the landfill) clearly clustered, at a similarity level of 50%; all these fingerprints had an intense band in common (see also below).

Table 1. Codes of the five clone libraries constructed from groundwater samples from the aquifer near Banisveld landfill*.

Code	Origin of sample	Pollution	Redox process	*Clones (n)*	*Geobacter* clones (%)	DGGE-types (n)	*Geobacter* types (%)
Fe_P1	In and directly underneath landfill	BEXN (221 g/l)	Fe (III)	37	32 (86)	13	10 (77)
Fe_P2	plume, 6m downstream	BN	Fe (III)	33	25 (76)	15	11 (73)
Fe_P3	plume, 21-39 m downstream	B	Fe (III)	40	33 (83)	13	9 (69)
Fe_C	beneath the plume, 39-58 m downstream	-	Fe (III)	47	30 (64)	17	11 (64)
NO_3_C	above the plume, 30-58 m downstream	-	NO_3^-	39	9 (23)	13	2 (15)

** Each clone library indicates from which samples it was constructed, as well as which type of pollution (B, benzene; E, ethylbenzene; X, xylene; N, naphthalene) was present and which redox process dominated, for more details see 'Site description' in 'Material and Methods' and reference 30. The samples used to construct the five clone libraries are also indicated by the ovals in Figure 1. In addition, results of screening the Geobacteraceae clone libraries constructed are listed: indicated are the numbers of clones screened per library, the number of confirmed Geobacteraceae clones, the number of different banding positions observed in DGGE ('DGGE types') and how many of these bands corresponded to Geobacteraceae are indicated.*

Groundwater samples taken at the same distance from the landfill body but at different depths in the plume were more similar to each other than to samples taken at other distances. The fingerprints from polluted groundwater samples close to the landfill (6-39 m downstream) were quite different from the DGGE profiles of the four polluted groundwater samples taken further downstream (48-78 m). The latter lacked the aforementioned highly intense band and clustered with samples from unpolluted iron-reducing (58c, 78c) and nitrate-reducing (48a, 58a, 68a, 78a) groundwater at the same distance from the landfill, at a similarity of 54%. Cluster analysis based on band absence or presence only, i.e. without

taking into account band intensity, failed to yield clearly separated groups of samples (less than 30% similarity (data not shown)).

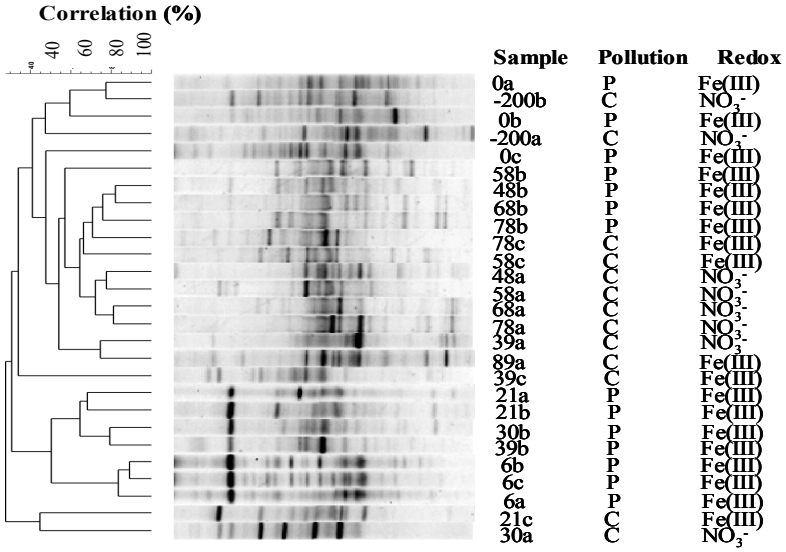

Figure 2. *UPGMA cluster analysis of DGGE profiles of Geobacteraceae (denaturant gradient: 30-55%). Sample codes are explained in the caption of Figure 1. The column marked 'pollution' indicates whether the analyzed groundwater sample was polluted (P) or clean (C), the column 'redox' indicates the dominant redox process (Fe (III) for iron reduction, NO_3^- for nitrate reduction).*

In order to relate the presence of individual DGGE bands to hydrochemical conditions, statistical analysis (Fisher's exact tests) was performed on 2 x 2 tables, containing the number of times a particular band was absent or present for a certain condition (pollution-level: clean vs. polluted, or redox-process: nitrate- vs. iron-reduction). The eight bands indicated in Figure 3 were found to be indicative for either pollution (significantly more present in polluted or clean groundwater, at $p<0.05$) or redox process (significantly more present in groundwater from nitrate or iron-reducing parts of the aquifer) (Table 2).

A distinctive and dominant band (band 2 in Figure 3) was observed in the DGGE profiles from polluted groundwater samples near the landfill that corresponded to iron-reducing conditions. This band was also observed for polluted groundwater samples further from the landfill, but at lower intensity (cf. lanes marked 48b-78b in Fig .3). It was absent from most clean groundwater samples, with the exception of samples –200b, 30a, and 78a (Figure 3). Fisher's exact test revealed that this band was significantly indicative of polluted groundwater ($p = 0.001$; Table 2). Also three other, less intense bands (bands 4, 5 and 8) were indicative of polluted groundwater. A plot of the relative intensity of the

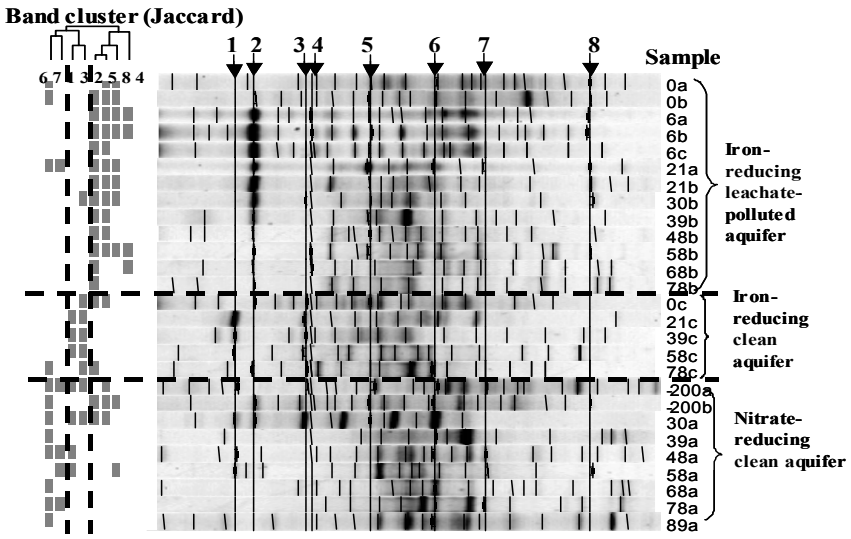

Figure 3. *Band-based analysis of the Geobacteraceae DGGE profiles. Numbered arrows indicate bands that are significantly ($p<0.05$) indicative of pollution level or redox process, as determined by Fisher's exact test (Table 2). On the left is a graphical representation of the occurrence of these indicator bands in the DGGE profiles. Band cluster analysis on this graphical representation was performed using the Jaccard coefficient.*

pollution-indicative bands in the DGGE profiles from plume samples, shows that bands 2 and 8 had the highest contribution to DGGE profiles in the first part of the aquifer, i.e., up to 39 m downstream of landfill (Figure 4A). In this part of the landfill also the concentrations of DOC and aromatic micropollutants decreased with increasing distance from the landfill (Figure 4B). In the second part of the plume, more than 39 m downstream of the landfill, the relative intensities of these bands were low and concentrations of DOC and micropollutants decreased little (Figure 4). By contrast, bands 1 and 3 were confined to groundwater samples from the clean part of the aquifer, although they did not appear in all clean samples (Figure 3, table 2). When analyzing the bands against redox process (one group containing groundwater samples from iron-reducing parts of the aquifer, and the other group containing groundwater samples from the nitrate-reducing parts of the aquifer), only two bands (bands 6 and 7; Table 2, Figure 3) significantly correlated with the occurrence of nitratereduction.

Phylogenetic analysis of *Geobacteraceae* communities

To obtain a more specific picture of *Geobacteraceae* diversity and of its relationship to hydrochemistry and biodegradation, phylogenetic analysis of cloned *Geobacteraceae* 16S rRNA genes was performed for five clone libraries, each of which was made for a composite groundwater sample that corresponded to a particular degree of pollution and redox condition (Table 1, Figure1). Three clone libraries (Fe_P1, Fe_P2, Fe_P3)

Table 2. Results from Fisher's exact tests on the relationship between presence of individual bands in DGGE profiles and environmental conditions*.

Polluted vs clean		**Iron reduction vs nitrate reduction**	
Band-position	p-value	Band-position	(p-value)
Band 1	0.006 (Clean)	Band 6	0.037 (NO_3)
Band 2	0.001 (Polluted)	Band 7	0.030 (NO_3)
Band 3	0.033 (Clean)		
Band 4	0.041 (Polluted)		
Band 5	0.006 (Polluted)		
Band 8	0.018 (Polluted)		

**DGGE* profiles *are shown in Figure 3. Bands that were significantly (p-value < 0.05) indicative of pollution level (clean vs polluted groundwater) are shown on the left, while bands significantly indicative of the dominant redox process (nitrate or iron reduction) are depicted on the right. For each band the significance (p-value) is indicated as well as (between brackets) for which specific environmental condition it was indicative.*

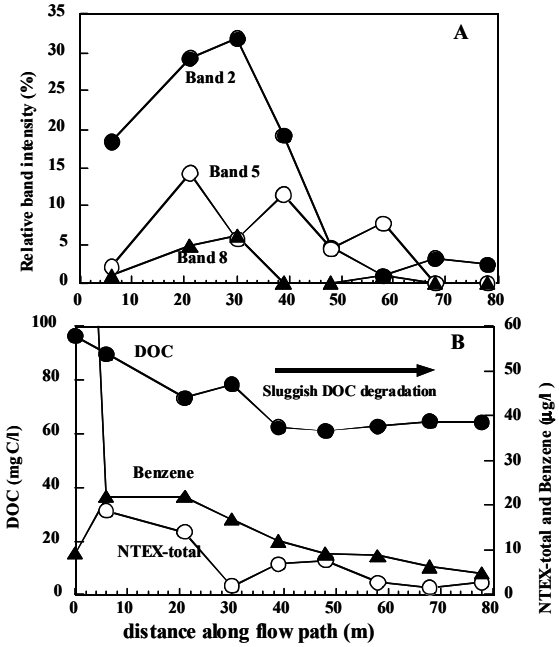

Figure 4. *Relations between occurrences of pollution-indicative DGGE bands and environmental conditions in the leachate plume from the Banisveld landfill. (A) changes in the intensity of indicator bands (●, band 2, ○, band 5 and ▲, band 8; Figure 3, Table 2), relative to the total intensity of the track, with distance along the flow path. (B) changes in concentrations of aromatic micropollutants and dissolved organic carbon (DOC) along the flow path (30). Average DOC (●) over 3 sampling events in 1998-1999, benzene (▲) and NTEX (Naphthalene, Toluene, Ethylbenzene, Xylene; ○) concentrations, determined in June 1998, were used for the plot.*

corresponded to parts of the leachate plume where organic pollutants are being degraded (29, 30), while clone libraries Fe_C and NO_3_C are reference clone libraries corresponding to the clean aquifer with iron-reduction and nitrate-reduction as dominant redox processes, respectively. Thirty-three to 47 clones per clone library were categorized based on migration behavior in DGGE, after which one representative per DGGE type was sequenced. A number of non-*Geobacteraceae* sequences and chimeras between *Geobacteraceae* and non-*Geobacteraceae* were detected. Chimeras were especially observed in the three clone libraries derived from the polluted aquifer (11 out of 12 chimeras observed). The non-*Geobacteraceae* sequences were mainly found in the clone libraries corresponding to the clean aquifer (16 out of 19 cases). The percentage of correct *Geobacteraceae* clones in the three clone libraries from the polluted iron-reducing aquifer was 76-86% (Table 1). This percentage was nearly four times higher than that observed for the clone library corresponding to groundwater taken from the nitrate-reducing, clean aquifer above the plume (23%). The higher percentage of *Geobacteraceae* clones recovered from the iron-reducing part of the aquifer parallels a higher number of *Geobacteraceae*-specific DGGE types (Table 1).

Geobacteraceae sequences were subjected to phylogenetic analysis (Figure 5). Most sequences from the three composite samples corresponding to polluted, iron-reducing parts of the aquifer, grouped together. They were most closely related to clones AY013645, AY013647 and AY013648, which had been isolated previously from the same polluted aquifer (Chapter 2, 22), and are only distantly related to cultured *Geobacter spp*. (<97.3% similarity). Clones Fe_P1-3, Fe_P2-17, Fe_P3-2 were highly identical (>99.1%) and showed a similar final migration position in DGGE, which corresponded to the dominant and distinctive band 2 in the DGGE profiles of groundwater *Geobacteraceae* (Figure 3, Table 2). Their DGGE positions were also similar to the previously obtained clones AY013647 and AY013648, which corresponded to the dominant band in general *Bacteria* DGGE profiles (Chapter 2, 22). While most clones coming from the polluted aquifer were most closely related to these previously retrieved clones, none of the 13 different DGGE types from the clean parts of the aquifer was.

The phylogenetic tree further reveals that the clones from the clean parts of the aquifer were more diverse with respect to 16S rRNA gene sequence than clones from the polluted part of the aquifer. The majority of clones fell into the genus *Geobacter*, but clones Fe_P2-10, Fe_P2-7, NO_3_C-2 and NO_3_C-138 were most closely related to the genus *Desulfuromonas* of the *Geobacteraceae.*

DISCUSSION

Geobacteraceae are widely distributed in metal-reducing environments (2, 5-7, 10, 11, 23, 25, 28) and are also associated with aromatic compound degradation (17, 23). However, detailed information about the relation between *Geobacter* community composition and

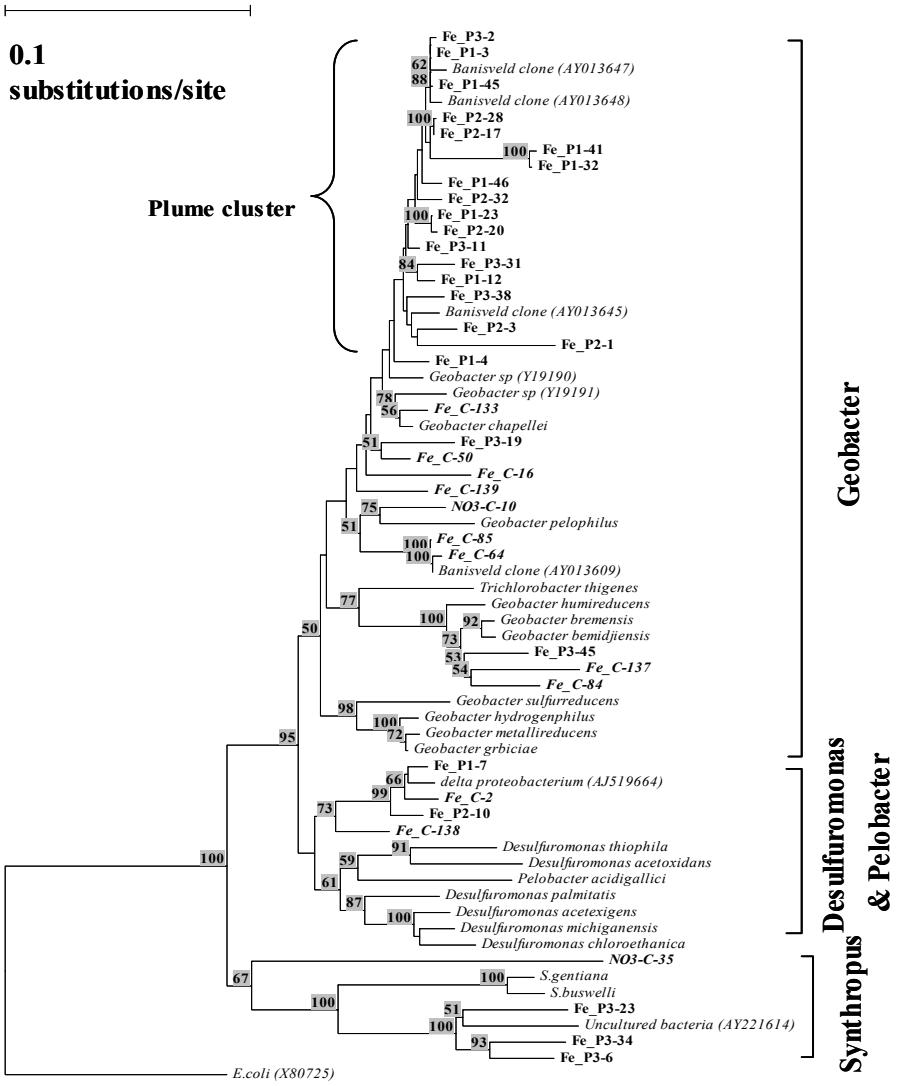

Figure 5. *Phylogenetic tree of Geobacteraceae clones from the Banisveld landfill leachate-polluted aquifer. A neighbor-joining analysis with Jukes-and-Cantor correction was performed on 737 unambiguous base positions. Only Bootstrap values above 50% are shown. Codes of the clones correspond to the sampling location from which the clones were derived and are explained in Table 1, the last number is clone-specific. All clones corresponding to the leachate plume are in bold, all clones from the unpolluted aquifer are in bold and italic.*

environmental conditions is scarce. Our results show that *Geobacteraceae* community composition in a landfill-leachate polluted aquifer corresponds to the level and type of pollution. They suggest that *Geobacteraceae* community composition may reflect biodegradation. A high *Geobacteraceae* diversity was observed, both outside and within the

plume of pollution. Care has to be taken in the interpretation of DGGE data because of the fact that the applied primer set is not totally specific for *Geobacteraceae*.

Specificity of applied primers and implications of the methodology used for data interpretation

The applied *Geobacteraceae*-specific PCR primer set did not exclusively amplify *Geobacteraceae* rRNA genes. Especially when the dominant redox process was not iron-reduction, a high number of non-*Geobacteraceae* sequences was observed. Previously, we observed that *Geobacteraceae* contributed to only a minor fraction (0.5%) of the microbial community in denitrifying groundwater (Chapter 2, 22). In the iron-reducing plume, *Geobacteraceae* contributed to 25% of the microbial community, based on both most-probable-number PCR and analysis of clone libraries constructed using *Bacteria*-specific primers (Chapter 2, 22). Therefore, the detection of high numbers of non-*Geobacteraceae* sequences seems to relate to the relative low abundance of *Geobacteracea*e in these samples. A second primer set for *Geobacteraceae*-specific PCR, amplifying *E. coli* 16S rRNA gene positions 494 to 825 (10), was employed to confirm the identity of *Geobacteraceae* clones, but it was found to amplify non-*Geobacteraceae* DNA fragments as well (data not shown). Other researchers have also reported the amplification of non-*Geobacteraceae* sequences when employing yet another *Geobacteraceae*-specific primer set (7). After aligning 16S rRNA gene sequences from cultured members of *Geobacteraceae* and the sequences retrieved in this study, we were not able to design primers that target *Geobacteracaea* more specifically (data not shown). In fact, primers *Geobacteraceae* 494F (10) and Geo564F (7), previously designed to detect *Geobacteraceae,* were found to contain a large number (>5) of mismatches and deletions towards more than 40% of the *Geobacteraceae* sequences retrieved in this study. We caution against concluding on *Geobacteraceae* diversity solely on the basis of the number of bands in '*Geobacteraceae*-specific' DGGE profiles, as this might well overestimate diversity due to presence of bands that do not correspond to *Geobacteraceae*. DGGE data need to be complemented by phylogenetic analysis, as was done in this study.

Phylogenetic analysis revealed a cluster containing 20 closely related sequences derived from the polluted aquifer. Microheterogeneity within a sequence cluster has also been observed by others (9, 13, 27), and also for *Geobacter* sequences (25). Microvariation can be partially due to artifacts introduced by PCR and cloning (26). In our study, the 20 clones constituting the sequence cluster gave rise to 18 different final migration positions in DGGE that all fitted with bands observed in the complex *Geobacteraceae* DGGE profiles directly generated from environmental samples (data not shown). A similar PCR-DGGE approach on DNA extracts from a single *Geobacter* strain and a mixture of 2 *Geobacter* species gave rise to 1 and 2 bands, respectively (data not shown), in agreement with what would be expected in the absence of PCR artifacts. Therefore, we conclude that PCR-

induced artifacts were not a major factor contributing to the microdiversity in *Geobacter* sequences.

PCR-based 16S rRNA gene analyses are also prone to other pitfalls (33). However, since the same experimental approach was applied to all samples and PCR-DGGE results were well reproducible (data not shown), all samples should have suffered from the same pitfalls, allowing between-sample comparison of the DGGE profiles.

***Geobacteraceae* community structure in relation to pollution and biodegradation**

Previous work on the same DNA extracts as employed in this study revealed that while cluster analysis of DGGE profiles obtained with general bacterial and archaeal primers discriminated between communities from polluted groundwater and clean water, it was not able to clearly distinguish between samples within the plume and to relate them to hydrochemistry and biodegradation (Chapter 2, 22). This study shows that community structures of the dominantly occurring *Geobacteraceae* are different within the plume: groundwater samples taken at 6 to 39 m from the landfill cluster together. They differ quite significantly from samples taken further downstream, which were more similar to the nearby non-polluted groundwater. This is not surprising since the aquifer close to the landfill has been exposed for the longest period of time to leachate and receives the highest concentrations of organic compounds, as well as the most reactive organic matter.

The clustering correlates with the observed disappearance of the micropollutants ethylbenzene, xylene and naphthalene over the first 39 m (29, 30). The biodegradation in the first part of the plume is also associated with high rates of iron reduction, presumably caused by the consumption of the more reactive DOC fraction there. This DOC (initial concentration 3.1 mM) is tenfold more reactive than a more persistent DOC fraction (6.1 mM) (29). Difference in iron oxide content in the polluted aquifer is unlikely to be an important factor that contributes to differences in the *Geobacteraceae* communities, as the content does not vary much along the flow-path (30).

The differences in *Geobacteraceae* community structure in the plume were largely reflected by an intense band in the DGGE profiles corresponding to samples taken close to the landfill (<48 m). Statistical analysis showed that this band, as well as 3 other less intense DGGE bands, is indicative for groundwater pollution. The band was absent from most DGGE profiles of clean groundwater and present in all DGGE profiles of plume samples. The band corresponded to a previously encountered sequence that also gave rise to the most intense band in DGGE profiles generated with *Bacteria*-specific primers and corresponded to 23% of the clones in a *Bacteria*-specific clone library (Chapter 2, 22). Its apparent selection by pollution, its high intensity in DGGE profiles suggesting high abundance, and its correlation with high iron reduction rates suggest that the Geobacters possessing this sequence play an important role in the attenuation of organic matter in the polluted aquifer.

Previously, Cummings *et al.* observed that metal contamination of Lake Coeur d'Alene sediments also selected for specific *Geobacteraceae* members (7). However, only 6 sediment cores were compared. As Cummings *et al.* discussed, their observations on *Geobacteraceae* distribution should be interpreted with caution, as many uncontrolled factors, such as different rivers functioning as source of inoculum, local heterogeneity and organic content, may also have influenced the *Geobacteraceae* distribution. In our study we analyzed 27 different groundwater samples from a well-characterized aquifer and were able to identify *Geobacteraceae* sequences that are significantly indicative for environmental conditions.

***Geobacteraceae* diversity as a factor in resilience to pollution**

The *Geobacteraceae* present in the iron-reducing plume differ from those in clean groundwater, even though the clean aquifer underneath the landfill leachate plume is also iron-reducing. A large diversity in *Geobacteraceae* was observed for the research location, especially in the clean, iron-reducing groundwater. Therefore, our analyses suggest that the Geobacters encountered in the plume were selected by the pollution, and have replaced the large pool of *Geobacter* species that had been present originally.

Other studies also revealed that phylotypes belonging to the *Geobacteraceae* can co-exist. Cummings *et al.* (7) obtained 4 to 9 different phylotypes for samples taken from a gradient of metal contaminants in Lake Coeur d'Alene. After *in situ* biostimulation of metal reduction in uranium-contaminated aquifers sediments, retrieved *Geobacteraceae* sequences could be grouped into two clusters (10). In benzene-oxidizing sediment from the petroleum-contaminated Bemidji aquifer three phylotypes were enriched (23). After benzoate amendment of the Borden aquifer eleven closely related *Geobacter* sequences were obtained, that differed from *Geobacteraceae* sequences retrieved from the unamended aquifer (25). These and our data suggest that generally species of the *Geobacteraceae* co-exist and form a pool of functionally redundant (with respect to iron-reduction) microorganisms. When environmental conditions change, such a pool may allow for a quick response.

A high diversity in *Geobacteraceae* in pristine aquifers might also be important for efficient biodegradation upon pollution: when more *Geobacter spp* with different physiological abilities are present, a larger number of organic compounds might be degraded. However, 16S rRNA gene based methods do not inform on the physiology of *Geobacteraceae.* Enrichment, isolation and physiological characterization are crucial for determining the functioning of *Geobacteraceae* in natural attenuation, for identifying the reasons behind the selection of particular *Geobacter* species upon organic pollution, and determining the potential of *Geobacteraceae* to respond to changes in environmental conditions, such as the depletion of certain iron oxides. In contrast to many other environmentally dominant microorganisms, Geobacters appear to be well-amenable to

cultivation (15, 25) and therefore we are currently attempting to isolate the dominant *Geobacter* species from the Banisveld landfill aquifer.

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Chapter 4

Culturable iron reducers in a landfill leachate-polluted aquifer

ABSTRACT

Using cultivation-independent methods we previously observed that Geobacter phylotypes dominated microbial communities in an iron-reducing aquifer polluted by the Banisveld landfill leachate. We now enriched for iron-reducing consortia using a range of media, with various electron donors and acceptors (i.e. sources of Fe (III)) and at various incubation conditions (pH, temperature, and presence or absence of the growth stimulator cAMP). In polluted sediments we detected 80-140 culturable iron-reducing bacteria per gram of sediment, five times more than in clean sediment. 16S rRNA gene analysis revealed that most phylotypes in iron-reducing enrichments were *Proteobacteria* (58 %). Others were *Firmcutes* (32 %) and *Bacteroidetes* (10 %). Despite applying various incubation conditions, the Geobacter that was dominant in terms of abundance in the cultivation-independent experiments, was not retrieved in the enrichments. Instead another *Geobacter* and iron reducers belonging to *Serratia* and *Clostridium* were isolated as pure cultures whilst *Rhodoferax* predominated in dilution-to-extinction environments.

INTRODUCTION

In soil, the oxidation of organic matter can be coupled to microbial iron reduction. (6, 11, 18-21, 23, 25, 43). A wide range of phylogenetically diverse isolates is capable of such iron-reduction, and these isolates are especially found within the gamma- and delta-division of the phylum of *Proteobacteria* (20). Within the *Deltaproteobacteria*, all members described to date of *Geobacteraceae* are able to conduct iron reduction (17). Geobacters appear to be important members of many metal-reducing subsurface environments: *Geobacter* species have been observed in a petroleum-contaminated aquifer (35), as well as in metal-polluted freshwater lake sediments (2, 7, 14, 15) and in a landfill leachate-polluted, iron-reducing aquifer (16, 31, Chapter 2, 3).

Iron reduction is the dominant redox process enabling the degradation of dissolved organic carbon (DOC), benzene (B), ethylbenzene (E), xylene (X) and naphthalene (N) in the aquifer polluted by leachate from the Banisveld landfill, The Netherlands (39). A clear difference was found between the microbial communities inside and outside the plume (Chapter 2, 31). Members belonging to the *Geobacteraceae* formed a considerable fraction of the microbial communities in the plume, contributing up to 25% to the cell counts (Chapter 2, 31). *Geobacteraceae*-specific community analysis revealed a relationship between the distribution of Geobacters and the degree of pollution. One *Geobacter* phylotype was found to dominate the part of the aquifer close to the landfill where attenuation of organic micropollutants and of dissolved organic matter occurred at relatively high rates (Chapter 3, 16).

The culture-independent methodologies of detecting microorganisms we used in these previous studies, do not necessarily give information on the functional potential of the 'living' soil. Non-abundant microorganisms may still be or no longer abundant microorganisms may have been responsible for the removal of pollutants they can utilize specifically, or electron acceptors only they can utilize. In this study therefore, we attempted to gain an overall insight into the culturable iron reducers present, using a range of media, with various electron donors and acceptors, and various incubation conditions (pH, temperature, and [cAMP](3, 4)).

MATERIALS AND METHODS

Site and hydrochemistry. The Banisveld-landfill research location is situated 5 km southwest of Boxtel, The Netherlands. Unlined landfilling with primarily household refuses occurred in a 6 m deep sand pit between 1965 and 1977. Hydrochemistry was characterized in 1998 and 1999 (39). Concentrations of dissolved organic matter, naphthalene (N) and the aromatic compounds benzene (B), ethylbenzene (E) and xylene (X) decreased within the plume along the groundwater flow direction (39). Iron reduction was the dominant redox process inside and beneath the plume, while nitrate reduction was observed above the

plume (see Figure 1 in (Chapter 2**,** 31)). More detailed information on the site and its hydrochemistry can be found in Van Breukelen et al (39) and Röling et al (Chapter 2, 31).

Sampling. In November 2002 and June 2003, sediment cores were taken anaerobically, transported and further treated as previously described (Chapter 2, 31). A sample from the polluted site was collected 21 m downstream of the landfill (2.75 to 3.15 m depth taken in 2002; 2.5-3 m taken in 2003). As a reference, a sample (of 2.3 to 3.2 m depth) was taken from an unpolluted reference location, 200 m to the west of the polluted aquifer.

Enrichment. Strictly anaerobic techniques were employed throughout the work. Modified basal medium (26) consisted of the following ingredients: (g l^{-1} distilled water): $NaHCO_3$, 2.5; NH_4Cl, 1.5; NaH_2PO_4, 0.6; KCl, 0.1; yeast extract, 0.01; $NaWO_4.2H_2O$, 0.00025; 10 ml trace element solution (medium 141, DSMZ), and 10 ml vitamin solution (medium 141, DSMZ). Iron-reducing enrichments were established by supplying different combinations of electron acceptors and donors to the modified basal medium. 10 mM iron pyrophosphate, 2.5 mM six-line ferrihydrite (F6L), 2.5 mM hematite, hematite plus 100 µM AQDS, or 10 mM iron citrate were supplied as electron acceptor and 2 mM acetate, 1 mM lactate, 0.5 mM benzoate or 0.001% yeast extract were supplied as electron donor (Table 1). F6L and hematite were supplied in a form of colloidal solutions at 2.5 mM (from 24 mM stock solution) prepared as previously described (34). 9 ml aliquots of media were into 10 ml serum bottles (Sigma-Aldrich, Germany) and anaerobic gas (N_2:CO_2=90:10) was bubbled through for 20 minutes. The bottles were then sealed by a butyl rubber stopper and an aluminum lid. After autoclaving and cooling, and $FeCl_2$ solution (to a final concentration 0.5 mM) was added as reductant to remove dissolved oxygen. Filter-sterilized six-line ferrihydrite, hematite colloidal solution, AQDS solution, toluene or benzene was added from stock solutions. 1 g sediments were added anaerobically to 9 ml of medium, followed by serial 10 fold dilution in the same medium down to a dilution of 10^{-5}.

Only the polluted part of the aquifer was sampled in June 2003 and used for enrichments. Ferrihydrite was used as sole electron acceptor in combination of different concentrations of the donor toluene (0.1, 0.5, 1.0 mM) or benzene (20 µM, 50 µM, 1.0 mM). The growth simulator cAMP (cyclic adenosine monophosphate; 10 µM final concentration) was added to medium containing acetate and ferrihydrite. Enrichments were also incubated at different temperatures (12 ^oC, 25 ^oC, and 30 ^oC) and pH (4.0, and 5.5). The detailed enrichment set-up is given in Table 1. 1 g of sediment was used as inoculum. Enrichment cultures were incubated at 25 ^oC in the dark, unless otherwise mentioned. The production of Fe (II) was monitored over time as described below.

Isolation. A modified roll serum bottle method (27) was used to isolate single colonies. The enrichment cultures were serially diluted into solid basal medium (at 50 ^oC), which was amended with 4 mM acetate, 20 mM iron Fe (III) citrate and 0.001% yeast extract.

Inoculated agar medium was incubated at 25 ^{o}C in the dark. Single colonies were picked up and transferred into basal medium containing Fe (III) citrate, acetate and small amount of yeat extract. That the isolates were iron reducers was confirmed by the measurement of Fe (II) production. Iron reducers were maintained by transferring the culture (1 % vol/vol) into the same medium. The purity of cultures was checked by DGGE profiling (see below).

Table 1. Media employed and the codes correspond to the ones in Figure 1 and 2.

Code[1]	Sample[4]	C-source (mM)[5]	Fe (III) source (mM)	Dil[7]	pH	T(^{o}C)
X-FePP-ace	Sed 2002	Acetate (2) + 0.001% YE	Fe (III) Pyrophosphate (10)	+	6.8	25
X-FePP-lac	Sed 2002	Lactate (1) + 0.001% YE	Fe (III) Pyrophosphate (10)	+	6.8	25
X-FePP-ben	Sed 2002	Benzoate(0.5)+0.001% YE	Fe (III) Pyrophosphate (10)	+	6.8	25
X-F6L	Sed 2002	Acetate (2) + Lactate (1) + 0.001% YE	6-line ferrihydrate (2.5)	+	6.8	25
X-Hem	Sed 2002	Acetate (2) + Lactate (1) + 0.001% YE	Hematite (2.5)	+	6.8	25
X-HemHum	Sed 2002	Acetate (2) + Lactate (1) + 0.001% YE	Hematite(2.5)+AQDS(0.1)[6]	+	6.8	25
P2-FeC	Sed 2003	Acetate (2) + 0.001% YE	Fe (III) citrate (10)	-	6.8	25
P2-FeC-pH4	Sed 2003	Acetate (2) + 0.001% YE	Fe (III) citrate (10)	-	4.0	25
P2-FeC-pH5.5	Sed 2003	Acetate (2) + 0.001% YE	Fe (III) citrate (10)	-	5.5	25
P2-FeC-12^{o}C	Sed 2003	Acetate (2) + 0.001% YE	Fe (III) citrate (10)	-	6.8	12
P2-FeC-30^{o}C	Sed 2003	Acetate (2) + 0.001% YE	Fe (III) citrate (10)	-	6.8	30
P2-F6L	Sed 2003	Acetate (2) + 0.001% YE	6-line ferrihydrate (2.5)	+	6.8	25
P2-F6L-AMP[2]	Sed 2003	Acetate (2) + 0.001% YE	6-line ferrihydrate (2.5)	+	6.8	25
P2-Hem	Sed 2003	Acetate (2) + 0.001% YE	Hematite (2.5)	+	6.8	25
P2-HemHum	Sed 2003	Acetate (2) + 0.001% YE	Hematite(2.5)+AQDS(0.1)[6]	+	6.8	25
P2-F6L-T	Sed 2003	Toluene(0.1-1)+0.001 YE	6-line ferrihydrate (2.5)	-	6.8	25
P2-F6L-B	Sed 2003	Benzene (0.02-1)+0.001% YE	6-line ferrihydrate (2.5)	-	6.8	25

1. X is either P or C; P, a sample from the polluted part of the aquifer; C, a sample from the clean part.
2. cAMP, 10 µM cAMP added.
3. Sed, sediment sample; the number refers to the year of sampling.
4. YE, yeast extract.
5. Electron shuttle
Dil, dilution-to-extinction applied; +, yes; -, no.

Physiological characterization of isolates. To evaluate the ability to use alternative electron acceptors, NO_3^- (10 mM), SO_4^{2-} (10 mM), Fe citrate (10 mM), amorphous ferrihydrite (10 mM), hematite (10 mM), lepidocrocite (α-FeOOH, 10 mM), goethite (γ-FeOOH, 10 mM), Fe phosphate (prepared at 1:1 PO_4/Fe (III) ratio, 10 mM), 10 mM AQDS or sulfur (20%) were added anaerobically to basal medium with acetate as electron donor (26). These media were inoculated (2% vol/vol) with a fully grown culture that had completely reduced the Fe (III) citrate used as electron acceptor.

To evaluate the utilization of different electron donors, glucose (3.0 mM), benzoate (0.5 mM), or H_2 was anaerobically supplied to the basal medium with F6L (2.5 mM) as the sole electron acceptor. Hydrogen was supplied by directly flushing the medium for 5 min. A washed cell suspension was used as inoculum (2 % vol/vol).

The lower and upper temperature limits for growth were tested using basal medium containing Fe (III) citrate (10 mM), and acetate (2 mM). Inoculated medium was incubated at 4, 12, 25, 30, 37, and 42 ^{o}C. pH tolerance for growth was tested using the same medium with pH set at 4.0, 5.0, 6.0, 7.0, 8.0 or 9.0. All inoculated media were incubated at 25 ^{o}C in the dark, unless otherwise mentioned.

DGGE profiling and analysis. DNA was extracted from 0.5 g sediment using the FastDNA spin kit for soil, following instructions of the manufacturer (Q.BIO-gene, USA). Cell suspensions were first treated with an equal volume of oxalate solution (containing per liter 28 g ammonium oxalate monohydrate and 15 g oxalic acid) to dissolve iron precipitates (26). After centrifugation, the cell pellet was suspended in sterile water, which was subsequently used for PCR. DGGE (denaturing gradient gel electrophoresis) profiling after 16S rRNA-gene based PCR revealed that the profiles with intact cells used as template in PCR were indistinguishable from the profiles using DNA extracted from those same cells (data not shown). Microbial communities in iron-reducing enrichments were profiled using DGGE of amplified 16S rRNA gene fragments and analyzed as described (Chapter 2, 31).

In order to determine the influence of addition of the growth stimulator cAMP on bacterial community structure in iron-reducing consortia, the similarity matrix was subjected to statistical analysis (non-parametric Kruskal-Wallis ANOVA tests), using Systat. 7.0, as described previously(41)

Sequencing and phylogenetic analysis. Bands cut out of DGGE gels or amplified 16S rRNA gene fragments of bacterial isolates were sequenced, and sequence analysis was performed as described previously (Chapter 2, 31). Both strands of 16S rRNA gene from *E. coli* position 8 to 1512 (for bacterial isolates) and 357 to 518 (for bands cut-out of DGGE gels) were sequenced. A phylogenetic tree for isolate NTA4 was constructed using sequences from *Geobacter bemidjiensis, G. bremensis, G. chapelleii, G. grbicium, G. humireducens, G. hydrogenophilus, G. metallireducens, G. pelophilus, G. sulfurreducens,* and the sequences of the dominant *Geobacter* phylotypes Fe_P1-3 (AY75273), Fe_P2-17 (AY752749) and Fe_P3-2 (AY752762), cloned previously from the polluted aquifer (Chapter 3, 16), while *E.coli*-X80 was used as an outgroup, i.e. a point on the tree corresponding to a much earlier time in the evolutionary history of those sequences.

Chemical measurements. Fe (II) was determined by ferrozine assay (42). The concentrations of nitrate and nitrite were estimated using Merkoquant test strips (Merck

GmbH, Germany). Sulfide was measured colorimetrically (37). The concentration of reduced AQDS was determined spectrophotometrically at 450 nm wavelength (22).

RESULTS

In an attempt to isolate representatives of the *Geobacter* phylotypes detected as dominant via previous culture-independent studies and with the aim of assessing the diversity and numbers of culturable iron reducers, sediment samples from the Banisveld landfill leachate-polluted aquifer were subjected to so-called dilution-to-extinction enrichments, i.e. aliquots of various dilutions were cultured under iron reducing conditions. A variety of conditions in terms of electron acceptors/donors, temperature, pH, and cAMP concentration were employed. Iron reduction in the various enrichments was confirmed by measuring the production of Fe (II) (data not shown). The number of culturable bacteria in iron-reducing media estimated by the MPN (most probable number) method (1) was approximately 80-140 (95 and 99% confidence limit respectively) per gram sediment from the polluted part of the aquifer, five times higher than the cell number in sediment from an unpolluted part of the aquifer (24 per gram sediment).

Bacterial community profiles in iron-reducing enrichments starting from polluted sediment (Figure1A) appeared to be more complex than those in enrichments inoculated with clean sediment (Figure1B). Enrichments containing the least diluted sediments, as well as an enrichment culture containing Fe (III) pyrophosphate and benzoate, and inoculated with a 10^{-3} dilution of sediment (code: P-FePP-Ben3, Figure 1A), fell into a cluster at 60% similarity. Within this cluster, a DGGE band of which the sequence was closest to a *Bacteroides* sequence was common to all samples. A small cluster consisted of enrichments containing more strongly diluted sediments, with Fe (III) pyrophosphate as electron acceptor and with lactate or acetate as electron donor, at 80% similarity (Figure 1A). The members of this cluster all contained a dominant phylotype most closely related to *Rhodoferax* (Figure1A). However, the DGGE profile of the environmental sample (coded P-sediment, Figure1A) with which the enrichments had been inoculated did not reveal the DGGE bands dominating the enrichments.

Relatively simple community profiles, with few bands, were observed in enrichment cultures receiving clean sediment as inoculum. Sequence analyses of three DGGE bands indicated that the sequences all belonged to the phylum *Proteobacteria* (Figure1B, Table. 2). A dominant species (band C-3, related to *Rhizobium*) was present in most of the enrichments from clean soil, and co-migrated with a faint band in the cultivation-independent DGGE profile of the sediment sample used for the enrichments (Figure1B).

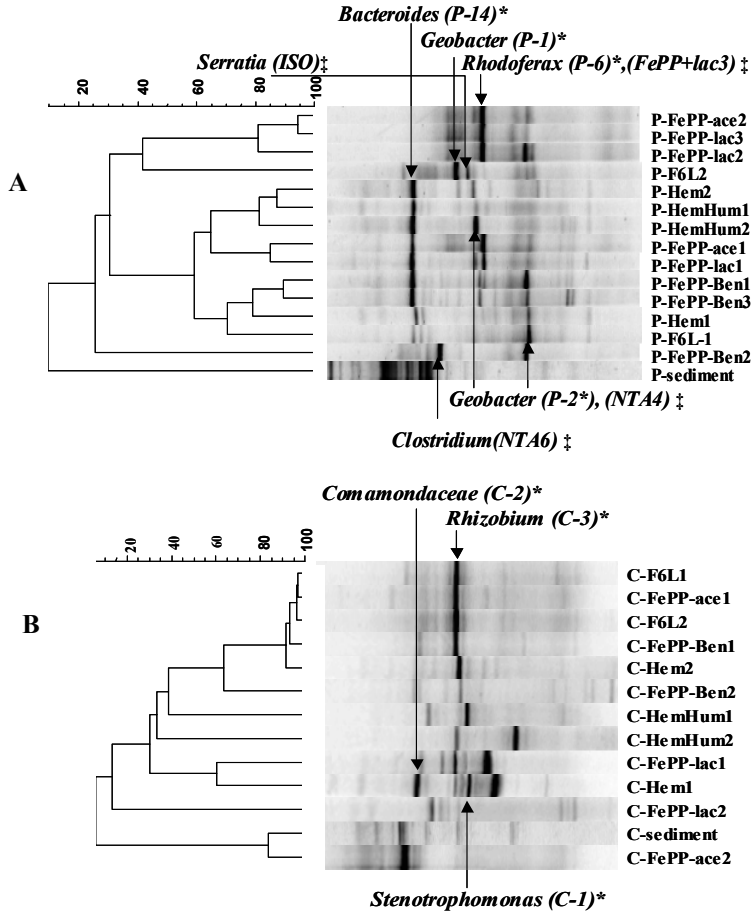

Figure 1. *UPGMA cluster analysis of DGGE profiles (30 to 55% denaturant gradient) of the bacterial community in iron-reducing enrichments containing different Fe (III) forms as sole electron acceptor, inoculated with, (**A**) landfill-leachate polluted sediment, or (**B**) clean sediments, obtained in November 2002. For the numbered bands, identities were obtained by analysis of partial (cutout bands, indicated by *) or the nearly complete 16S rRNA gene of the isolates (‡) and shown in Table 2. Characters behind each DGGE profile represent sediment origin (P: polluted; C: clean) - electron acceptor (FePP: Fe (III) pyrophosphate; F6L: 6-line ferrihydrite; Hem: hematite; Hum: humic acid analog (AQDS)) – electron donor (lac: lactate; ace: acetate; Ben: benzoate), and dilution factor (1: 10^{-1}; 2: 10^{-2}). More information can be obtained in Table 1.*

Enrichment cultures coded P-FePP-Ben3, P-FePP-Ben2 and P-F6L-2 recovered from polluted sediments differed in terms of their dominant bacterial species (Figure 1A) and were therefore selected for isolation of pure cultures of iron reducers and diluted into agar medium. Isolates that were able to reduce Fe (III) were subjected to phylogenetic analysis by sequencing their 16S rRNA genes. Three isolates for which the banding position of 16S rRNA gene fragments in DGGE corresponded to dominant bands in the enrichments from which they were retrieved, and which were capable of reducing Fe (III), were identified as *Geobacter sp* (indicated by code NTA4 in Table 2 and Fig 1A, 95%

similarity to AF523968), *Serratia proteamaculans* (ISO, 99% similarity with AY040208) and *Clostridium sp* (NTA6, 99% similarity with AY221993) (Table 2). Phylogenetic analysis placed isolate NTA4 into the *Geobacteraceae,* but revealed that its sequence was quite different from the *Geobacter* sequences [Fe_P1-3 (AY75273), Fe_P2-17 (AY752749) and Fe_P3-2 (AY752762)] dominating our previous, cultivation-independent study (Figure 3 in Chapter 3, 16).

In attempts to enrich and isolate the dominant *Geobacter* (Chapter 3, 16), additional incubations with a variety of electron donors and acceptors and with various incubation conditions were carried out with sediment samples taken in June 2003 from the polluted aquifer. A more complex community profile was found as compared to enrichments containing sediment taken in November, 2002 (Figure 2). In order to obtain higher numbers of culturable iron reducers, cAMP, a compound that has been reported to enhance the culturability of heterotrophic bacteria was added to the growth media (3, 4). However, addition of cAMP neither increased the number of culturable iron reducers significantly, nor did it affect the community structure ($p>0.05$), enrich the dominant *Geobacter* observed in the earlier culture-independent study (Chapter 3, 16). As shown in Figure 2, the two samples to which cAMP had been added clustered at 55% similarity.

Iron-reducing enrichments were also established with the pollutants toluene and benzene as potential electron donors. However, whether these enriched cultures actually degraded toluene or benzene, or utilized other organic compounds (i.e. the added yeast extract, 0.001%) instead has not been evaluated. Enrichments with toluene and benzene clustered at a similarity of 65% with one exception, the enrichment containing ferrihydrite and the highest concentration of benzene (1 mM) and inoculated with a 10^{-3} dilution of sediment (code: P2-F6L-B3) (Figure 3). Within this group, most of the samples had some DGGE bands in common, relating to *Bacillus* (coded P-9, see also Table 2), *Serratia* (P-3, P-4) and *Firmicutes* (P-11). These sequences and DGGE banding position, were not frequently encountered for other consortia (Table 2, Figure 2). PCR amplification on the enrichment with 50 μM benzene did not yield sufficient PCR product for analyses by DGGE.

Consortia obtained under different incubation conditions (temperature and pH), did not contain the dominantly occurring *Geobacter* phylotype (Figure 1A, 2). Enrichments at 12^{o}C and 30^{o}C contained a *Serratia* species (band P-4, Figure 2, table 2). *Duganella* (P-8) was also enriched at 12^{o}C. Species belonging to *Anaerospora* (P-10), *Firmcutes* (P-11), and *Geobacillus* (P-12) dominated in the enrichment incubated at 25^{o}C. The DGGE profile of the pH 4.0 consortium was more complex than that of the pH 5.5 consortium. Neither consortium contained the *Serratia* (P-3, 4), or *Bacteroides* (P-15) that were common in consortia with Fe (III) oxides as electron acceptor (i.e. Hematite or F6L).

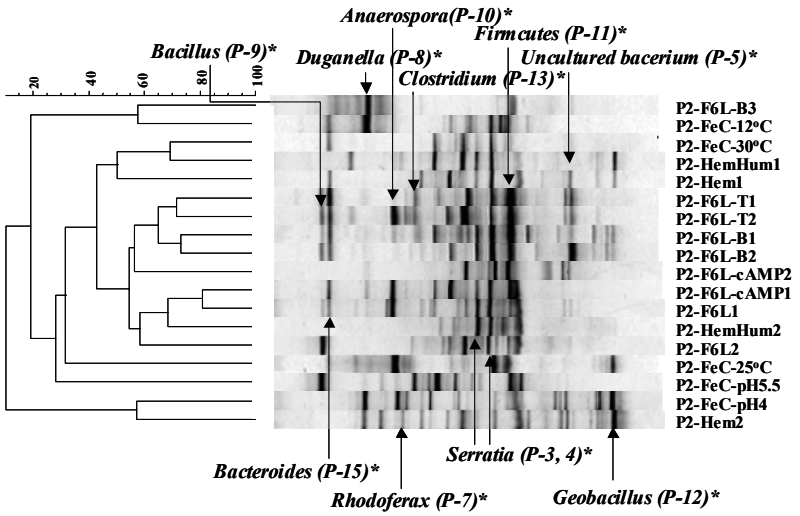

Figure 2. *UPGMA cluster analysis of DGGE profiles (30 to 55% denaturant gradient) of the bacterial community in iron-reducing enrichments containing different Fe (III) forms as sole electron acceptor, inoculated with landfill-leachate polluted sediment sampled in June 2003. For the numbered bands, identities were obtained by analysis of partial 16S rRNA gene of cutout bands, indicated by * and shown in Table 2. Characters behind each DGGE profile represent sediment origin (P2: polluted sediment sampled in June 2003), electron acceptor (FeC: Fe (III) citrate; F6L: 6-line ferrihydrite; Hem: hematite; Hum: humic acid analog (AQDS)), B: benzene; T: toluene; dilution factor (1: 10^{-1}; 2: 10^{-2}); cAMP: cyclic AMP; and different incubation conditions: 12°C, 25°C and 30°C; pH 4.0 and pH 5.5. More information can be obtained in Table 1.*

Geobacter NTA4 was evaluated in terms of its utilization of alternative electron acceptors and donors, and its tolerance to pH and temperature variation. The isolate can reduce different types of iron oxides (amorphous ferrihydrite, lepidocrocite and goethite (γ-FeOOH)) and chelated Fe (III) (Fe citrate and Fe phosphate). The isolate also reduced AQDS, nitrate, sulfur, but it did not reduce sulfate. Glucose and H_2 were used as electron donors for iron reduction. Iron reduction was observed between 4 and 30°C, and at pH's ranging from 6 to 8 (data not shown).

DISCUSSION

Previous cultivation-independent studies indicated that Geobacters contributed up to 25 % of the cell counts in the iron-reducing landfill leachate plume, and that the distribution of Geobacters correlated with the level of pollution (16, 31, Chapter 2, 3). The clustering of *Geobacter*-specific community profiles and the dominance of a single DGGE band (dominant *Geobacter*) within these profiles corresponded to the part of the aquifer where organic micropollutants were attenuated at relatively high rates (Chapter 3, 16).

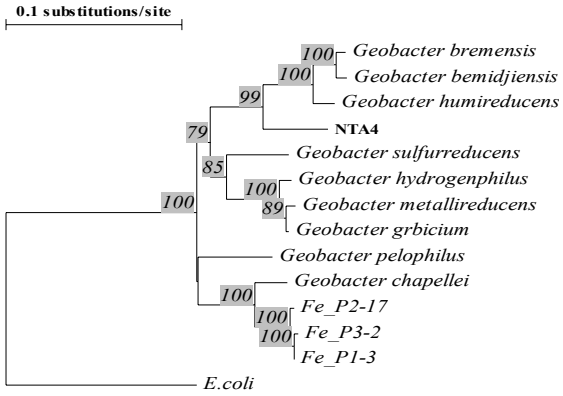

Figure 3. *Phylogenetic placement of the Geobacter strain NTA4 isolated from the Banisveld landfill leachate-polluted aquifer. A neighbor-joining analysis with Jukes-and-Cantor correction was performed on 730 unambiguous base positions. Only Bootstrap values above 70% are shown.*

It is often claimed that *Geobacter* species are readily culturable (24), in contrast to many other microorganisms that are strongly contributing to microbial communities and have significant effects on the pollution of environments. This was the main reason for us to attempt to isolate the *Geobacter* species the 16S rRNA gene of which strongly contributed to clone libraries prepared on the basis of soil samples (16, 31, Chapter 2, 3). However, despite the use of a wide range of incubation conditions (with respect to pH, temperature and sources of Fe (III) and carbon) we were unable to enrich these numerically important Geobacters.

Instead, other genera and *Geobacter* species were enriched and isolated, indicating the applied media and conditions were suitable for recovery of members belonging to the *Geobacteraceae* but apparently not for the *Geobacter* strain of interest. We recovered two phylotypes and one isolate affiliated with *Geobacter,* however the proportion of culturable Geobacters (approximately 10^2 cell/g sediment) was quite low compared to the observation that in a cultivation-independent study Geobacters contributed 25 % of the cell counts (Röling et al. 2001), which are in the order of 10^6 cells/ml groundwater at our research location (Röling, unpublished). In addition, efforts to recover the dominant *Geobacter* from groundwater samples, were not successful either; only iron-reducing members belonging to *Desulfitobacterium* and *Clostridia* were found (data not shown). Rooney-Varga et al (32) were not able to retrieve Geobacters in pure culture from an iron-reducing, benzene degrading enrichment in which *Geobacter* was a dominant member of the microbial community either. This suggests that members of the *Geobacteraceae* that dominate the soil of interest may be less readily culturable than suggested (Lovley et al. 2004). Perhaps they require conditions that are closer to those in their natural habitat than any of the wide variety of conditions we offered. A similar phenomenon was encountered for another study

location with a comparable environmental conditions as ours: *Geobacteraceae* phylotypes dominated microbial communities in the sediment, however, the numbers of culturable iron reducers were only 240 cells ml^{-1}, very low compared to the 1.5-8 x 10^7 cells/ml determined by direct counting (30).

Table 2. 16S rRNA gene based identities of cut-out sequences and isolates relating to coded bands in DGGE profiles of iron-reducing enrichments (Figure1, 2).

Code	Closest relative in Genebank	Phylogenetic group	Accession no.	Similarity %	Size of sequenced 16S rRNA
NTA4 (isolate)	*Geobacter* sp	δ- Proteobacteria	AF523968	95	713
P-1	*Geobacter psychrophilus*	δ- Proteobacteria	AY455853	99	83
P-2	*Geobacter* sp	δ- Proteobacteria	AF019937	98	151
ISO (isolate)	*Serratia proteamaculans*	γ-Proteobacteria	AY040208	99	756
P-3	*Serratia* sp	γ-Proteobacteria	AY745744	94	127
P-4	*Serratia* sp	γ-Proteobacteria	AF427159	95	171
C-1	*Stenotrophomonas maltophilia*	γ-Proteobacteria	AY472115	100	195
P-5	Uncultured bacterium	γ-Proteobacteria	AF257844	97	45
FePP+lac3 (isolate)	*Rhodoferax ferrireducens*	β-Proteobacteria	AF435948	99	1448
C-2	*Comamondaceae*	β-Proteobacteria	AJ505858	100	197
P-6	*Rhodoferax* sp	β-Proteobacteria	AF435948	98	155
P-7	*Rhodoferax* sp	β-Proteobacteria	AY788965	100	116
P-8	*Duganella violaceinigra*	β-Proteobacteria	AY376163	99	113
C-3	*Rhizobium*	α-Proteobacteria	AJ619085	98	170
NTA6 (isolate)	*Clostridium* sp	Firmicutes	AY221993	99	1386
P-9	*Bacillus* sp	Firmicutes	AY803983	97	154
P-10	*Anaeroospora* sp	Firmicutes	AY731461	97	130
P-11	Uncultured firmicutes clone	Firmicutes	AY406552	92	89
P-12	*Geobacillus kaue*	Firmicutes	AY608975	95	145
P-13	*Clostridium celerecrescens*	Firmicutes	AY458859	100	148
P-14	*Bacteroides*	Bacteroidetes	AJ534683	99	124
P-15	*Bacteioides*	Bacteroidetes	AY144266	95	147

Cultivation revealed that besides Geobacters other iron reducers are present in the aquifer. These iron-reducers belong to *Serratia, Clostridium* and *Rhodoferax.* However, these culturable iron reducers formed only approximately 0.01% of total bacteria counts before cultivation (Chapter 2, 31). Previously, it was found that a *Serratia marcescens*

strain was able to reduce hematite with glucose plus asparagine as electron donor (28). In this study, also an iron-reducing *Serratia* isolate was obtained. *Serratia* may have an ecological advantage over other microorganisms in that it can produce prodigiosin, a compound toxic to protozoa (13). The eukaryotic communities in the leachate-polluted sediment near the Banisveld landfill are dominated by protozoa (Brad and Röling, manuscript in preparation). *Serratia* may also contribute to the control of the protozoa population as production of prodigiosin may limit the growth of protozoa.

The *Clostridium* isolates that were capable of dissimilatory Fe (III) reduction, probably used Fe (III) as an electron sink as reported for *Clostridium beijerinckii* and *Clostridium butyricum* (8, 29). *Clostridium* are often observed at landfill sites (5, 40), however, the proportion of *Clostridium* in the aquifer polluted by the Banisveld landfill is likely to be small as the species was not encountered in clone libraries (Chapter 2, 31).

Rhodoferax ferrireducens is a facultatively anaerobic bacterium which can reduce Fe (III) coupled to the oxidation of acetate (10). Interestingly, phylotypes affiliated to *Rhodoferax* were also dominant in iron-reducing enrichments originating from diesel-contaminated groundwater, and a retrieved *Rhodoferax ferrireducens* strain was capable of degrading propylbenzene to propylphenol coupled to iron reduction (9). A *Rhodoferax*-related sequence was previously found in a clone library derived from leachate-polluted groundwater downstream of the Banisveld landfill (Chapter 2, 31), but that sequence [(96% similarity to *R. fermentans* (D16211)] was different from the sequences retrieved in the present study [99% similarity to *R. ferrireducens (*AF435948) for isolate FePP-lac3, 98% to *Rhodoferax sp* (AF435948) for DGGE band coded P-6 and 100% to *Rhodoferax sp* (AY376163) for band coded P-7] suggesting diversity within *Rhodoferax* in Banisveld landfill.

In the present study*, Bacteroides*-like species were predominantly present in the iron-reducing enrichments (Figure 1A, 2) and indeed they have been found before in other iron-reducing environments (33, 38, chapter 5), as well as in anaerobic landfills (12). Whether *Bacteroides* species are able to dissimilate Fe (III) is unknown. They probably ferment complex organic matter and supply the iron reducers with simple organic compounds, i.e., lactate and acetate, or lower the redox potential, which is necessary for iron reducers to function properly (36).

Previously, it was reported that in the aquifer we study, selection of microbial species occurs, under the influence of pollution (16, 31, Chapter 2, 3). In the present study, higher numbers of culturable microbes and more complex community profiles were observed in the iron-reducing enrichments from polluted sediment than in those containing clean sediment. Geobacters were enriched from the polluted sediment but not from the clean sediment.

In summary, this cultivation-based study revealed that there are other iron reducers besides *Geobacter* in the Banisveld landfill-impacted aquifer. The dominant *Geobacter* is

not readily obtained in pure culture. It also revealed an appreciable flexibility in the use of electron acceptors by isolate *Geobacter* NTA4.

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Chapter 5

Diverse dissimilatory Fe (III) reducers and their metabolic response to environmental conditions in the Scheldt estuary

ABSTRACT

In intertidal sedimentary environments where sulfide is produced, it is hard to distinguish between the abiotic and the biotic processes contributing to iron-reduction. In order to address the microbial potential for iron reduction, we combined field-scale geochemical measurements with laboratory experiments on the associated microbiology. We did this both for a freshwater (Appels) location and a brackish (Waarde) location in the Scheldt estuary, Northwest-Europe. At both locations dissolved Fe (II) abounded in pore water. Ascorbate-extractable, bioavailable Fe (III) phosphate was ample at all depths sampled. There were nearly 30 times higher concentrations of dissolved sulfate and sulfur in pore water at the Waarde site as compared to Appels with a rapid increase of sulfate concentration from 10 to 25 cm depth for both sites, suggesting that the activity of sulfate reduction at Waarde was higher than Appels. Microbial community structure was analyzed by a combination of cultivation-independent 16S rRNA gene analysis, and enrichment, strain isolation and physiological characterization. Dilution-to-extinction enrichments with a variety of Fe (III) sources were performed, as well as retentostat enrichments. The dilution factor of the inoculum used for enrichment mainly determined the iron-reducing microbial community structure, much more so than the source of Fe (III). Well-known iron-reducers, such as members of the family *Geobacteraceae* and of the genus *Shewanella*, constituted only a minor fraction of the microbial community. Instead, facultative anaerobic iron-reducing *Ralstonia* and strictly anaerobic, spore-forming *Clostridium* dominated. These species were flexible in the use of electron acceptors and donors, and grew at a wide range of temperatures and pH. These findings, the abundance of culturable iron reducers (4.6×10^5 and 2.4×10^4 cells g^{-1} sediment for Appels and Waarde, respectively), as well as the substantial bioavailability of sources of Fe (III), suggest that there is a high potential for microbiological iron reduction in Scheldt estuarine sediments. The observed composition and properties of the microbial communities is discussed in relation to the dynamic conditions in the Scheldt estuary.

INTRODUCTION

Iron reduction is an important biogeochemical process in many anaerobic environments. Fe (III) can be reduced abiotically by a variety of reductants, for instance by sulfide in sulfate-reducing intertidal mud flats (27, 35). In the past, abiotic iron reduction was considered the main or even sole cause of Fe (II) formation (42, 81). However, abiotic iron reduction is no longer considered to dominate environmental iron reduction, not even in sulfide-producing environments. Instead, enzymatic iron-reduction appears to be a ubiquitous and important redox process in marine sediments (8, 20, 30) and freshwater sediments (13, 32, 70, 73). Besides their role in the biogeochemical cycling of iron in marine and freshwater sediments (50), iron-reducing microorganisms are also actively involved in the biogeochemical cycling of other metals (2, 12, 21), as well as in the oxidization of as debris from plants and anthropogenic organic matter generated in industrial and agricultural activities (29, 56).

Microorganisms able to carry out enzymatic iron reduction are phylogenetically diverse; they are found throughout the *Bacteria* and *Archaea* (39). The *Proteobacteria* contain a large proportion of the currently known iron reducers, such as *Shewanella, Geobacter* and *Anaeromyxobacter* (42, 54). Particularly all presently characterized members from the *Geobacteraceae,* affiliated to the *Delta-proteobacteria*, are capable of dissimilatory Fe (III) iron reduction to support their growth, with ATP formation resulting from oxidative phosphorylation by electron transport along an electron transport chain (38). Other iron reducers, such as those belonging to the fermentative *Firmicutes* ((10, 53) just use Fe (III) as an electron sink for the oxidation of NADH, and in this way are able to use metabolic pathways allowing for ATP formation via substrate-level phosphorylation.. Geobacters are frequently found in anaerobic subsurface environments (2, 9, 22, 37, 51, 55, 62, Chapter 2, 3) while facultative anaerobic *Shewanella* have been recovered from shallow subsurface environments (42). However, they did not dominate salty marsh sediments, such as on Sapelo Island (45).

The location studied in this paper, i.e. the Scheldt estuary in North-Western Europe, consists of freshwater rivers feeding into an estuary where the freshwater is mixed with seawater under tidal influences, leading to a gradient of salinity. Sediments in the Scheldt estuary have been impacted by the entry of wastewater from the cities of Antwerp and Brussels (1.5 million people), located in one of the most densely populated areas in the world. The estuary is also affected by other agricultural, municipal and industrial activities (63). The Scheldt river has a total length of 355 km and flows through France, Belgium and The Netherlands. The Scheldt estuary receives about 10^5 tons of organic, largely anthropogenic, carbon annually (79). The tidal exchange (100000 m^3 s^{-1}) is much higher than the average river discharge (120 m^3 s^{-1}), resulting in a residence time of up to 2-3 months for the highly polluted freshwater (5). The large input and accumulation of organic matter leads to high microbial activity and oxygen depletion in the upper layer (0.3-0.5 cm deep) of sediments (6, 19, 36, 79). Vertical variation in redox zonation and changing redox

conditions were observed in estuarine sediments (69, 82). Nitrate reduction, sulfate reduction and methanogenesis have been reported (23, 36, 52). Abiotic iron reduction due to microbes-produced sulfide in the Scheldt estuarine sediments was observed (23, 24, 52, 82), however, how far enzymatic iron-reduction plays a role in iron reduction and which if any microorganisms perform this enzymatic activity is unknown.

This study addresses the potential for enzymatic iron reduction for two locations, freshwater Appels (Belgium) and brackish Waarde (The Netherlands), both in the Scheldt estuary, via field-scale geochemical measurements combined with laboratory experiments on the associated microbiology. The community structure was determined in environmental samples and in enrichment cultures thereof, in its dependence on the types of iron oxides present and on the concentration at which the sediments were inoculated. Through phylogenetic analysis and physiological characterization, insight was obtained into the iron-reducing microbial communities.

MATERIALS AND METHODS

Site description. The two research locations, Appels and Waarde are intertidal marsh sites, located in the Scheldt estuary (Figure 1). The mean pore water (i.e. the water filling the spaces between the grains of sediment) salinities measured at Appels and Waarde were 0.78 ± 0.15 PSU (practical salinity units) and 10.92 ± 1.37 PSU, respectively. The Appels intertidal site, 127 km upstream of Vlissingen in the freshwater upper estuary, is situated in the outside bend of a river meander. The upper marsh is flooded only during exceptionally high tides and is vegetated by willows, while the lower mudflat is vegetated by rush reed and is flooded more frequently. Samples were collected from the lower mudflat, which is not vegetated and flooded at each tidal cycle.

The intertidal marsh at Waarde is located 40 km upstream from Vlissingen, in the brackish part of the lower estuary. Vegetation consists mainly of *Spartina* species (*Aster* and *Puccinellia*) at the marsh edge. The sediment exhibits a vertical succession of silt and sand layers. Macrofauna containing mainly oligochaete worms abounds and contributes intense bioturbation and bioirrigation to the Appels site, whereas there is only a moderate density of oligochaetes and benthic macrofaunal nematode and polychaetes at the brackish Waarde site (61).

Sampling and analysis of geochemical parameters. Sampling was performed in February 2001 for intertidal water and solid phase analysis. Pore waters were sampled using pore water diffusion equilibrators (”peepers”) as described (23). In the field, under anaerobic condition, pore waters were extracted and filtered through 0.2 μm pore size Whatman Puradisc polypropylene filter. 1 ml pore water was used for pH measurements, while 1 ml was immediately diluted with 9 ml of MilliQ water, acidified with nitric acid, for later measurement of dissolved S and Fe by ICP/OES (Inductively Coupled Plasma/Optical

Emission Spectroscopy). Sulfate was also determined by Ion Chromatography on samples preserved in 0.05 N HCl.

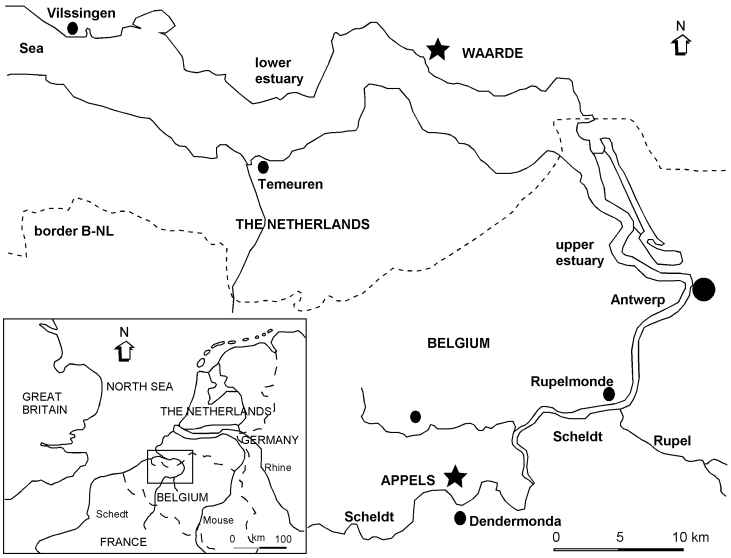

Figure 1. *Map of the Scheldt estuary indicating the research locations Appels and Waarde. The Appels site is located in a freshwater tidal river, which extends upstream from the Scheldt river. Waarde is located in the brackish part of the estuary. The part of the estuary between the Belgian-Dutch border and the North Sea is also known as the Western Scheldt (Westerschelde).*

Sediment for solid phase characterization was collected using a stainless steel wedge corer (25), and sectioned into 1 cm slices in a glove box under N_2 atmosphere, and stored in sterile plastic bags at -30 ^oC. After thawing and homogenization, sediment samples were mixed with an ascorbate solution in one-step batch extractions (34), treated by a mixture of HF and $HClO_4$: HNO_3 (3:2) at 90 ^oC for 12 hours, dried at 150 ^oC for 4 hours (23). The concentrations of S and Fe in the ascorbate extract were measured by ICP/OES.

Sampling for microbiological characterization. In November 2002, sediment samples were taken anaerobically using ethanol-sterilised PVC pipes (25 cm in length, 5.5 cm in diameter). Duplicate sediment cores, at 1meter distances, were collected. The sediment cores were transferred immediately into an anaerobic jar containing oxygen scavenger (AnaeroGen^{TM}, Oxoid Limited, England). One sediment core was used for batch enrichments on the day of sampling, the other core was stored at –20 ^oC for later molecular analysis. For batch enrichments, the sediment between 5 and 15 cm below the surface was subsampled within an anaerobic glove box. Sediment cores for molecular characterization

were cut with an ethanol-sterilized saw in order to obtain sediment fractions corresponding to 0-3, 3-6, 6-9, 9-12, and 12-15 cm depth.

Batch enrichment and isolation. Standard, strictly anaerobic techniques were employed throughout the operation. Modified basal medium (43) consisting of the following ingredients (g l^{-1} distilled water) $NaHCO_3$, 2.5; NH_4Cl, 1.5; NaH_2PO_4, 0.6; KCl, 0.1; $NaWO_4.2H_2O$, 0.00025; 10 ml trace element solution (medium 141, DSMZ), and 10 ml vitamin solution (medium 141, DSMZ) was amended with 2 mM acetate, 1 mM lactate, and 0.01 % yeast extract. 12 g l^{-1} NaCl was added to the medium in case of enrichments from the brackish Waarde sediments; this concentration corresponds to the *in situ* NaCl concentration at Waarde. Four types of enrichments were set-up: basal medium was supplied with chelated Fe (III) (10 mM Fe (III) citrate), amorphous iron oxide (Fe_2O_3; 2.5 mM six-line ferrihydrite, F6L), crystalline iron oxide (Fe_2O_3) (2.5 mM hematite), or 2.5 mM hematite plus 100 μM electron shuttle [humic acid analog, 1,6-anthraquinone disulfonate (AQDS)]. F6L and hematite were supplied in a form of colloidal solutions, prepared as previously described (60). After dividing the media over serum bottles, the media were flushed with anaerobic gas (N_2:CO_2=90:10) for 20 minutes and sealed with butyl rubber stoppers. After autoclaving and cooling, $FeCl_2$ solution was added as an oxygen scavenger (0.5 mM final concentration) and filter-sterilized six-line ferrihydrite colloidal solution, hematite colloidal solution or AQDS solution was added to the media. Batches were inoculated in an anaerobic glove box (Plas-labs, Lansing, MI, USA) under an atmosphere of N_2:CO_2 (90:10%) gas. 10 g of sediment (corresponding to 5 to 15 cm depth) was well mixed with 90 ml 0.1% sodium pyrophosphate by shaking for 5 minutes in order to release the sediment-attached bacteria. One ml of sediment suspension was inoculated into 9 ml of basal medium, followed by 10-fold serial dilutions in the same medium up to a dilution of 10^{-7}. These enrichment cultures were incubated at 30 ^oC in the dark and positive enrichments (as judged from measurement of produced Fe (II) and color change due to ferric iron reduction) were maintained by transferring them to fresh medium with a composition corresponding to the medium of the original enrichment.

A modified roll serum bottle method (48) was used to isolate single colonies from selected enrichments, after three transfers. These cultures were serially diluted into basal medium that was supplemented with 1.5 % agar (at 50 ^oC), and contained 4 mM acetate, 2 mM lactate and 20 mM iron citrate. The bottles were incubated at 30 ^oC in the dark. Single colonies were picked up and transferred into basal medium with Fe (III) citrate, lactate, and acetate at neutral pH. Whether the isolates were iron reducers was confirmed by the measurement of Fe (II) production; a strain was considered an iron reducer when the amount of iron produced was at least twice as much as the Fe (II) concentration in uninoculated medium. The culture's purity was checked by its DGGE profile of amplified 16S rRNA gene (see below). Iron reducers were maintained by transferring the culture (1% vol/vol) in the same medium. Numbers of culturable iron-reducing bacteria in enrichments

were estimated by the MPN (most probable number) approach (1). In this case, enrichments containing 10-fold serial dilution of sediments with hematite plus humic acid analog (AQDS), six-line ferrihydrite or iron citrate as sole electron acceptor were considered as triplicate treatments.

(Semi-) continuous culturing enrichment. A (semi-) continuous culturing approach was used to enrich for slow-growing iron reducers, starting with a continuous culturing approach with biomass retention (retentostat) that was later shifted to the chemostat mode (continuous culturing without biomass retention). The retentostat reactor was built as previously described (59), with a working volume of 1.8 liter controlled by means of a liquid-level indicator and a retention unit (containing a 0.22 μm pore size filter), both inserted through the top plate of the fermentor. The liquid-level indicator regulated a peristaltic pump that kept the volume of the fermentor constant by withdrawing liquid from the culture through the retention unit. Agitation was obtained by flat-blade, propeller-type impellers operating at 200 rpm. Culture pH (6.8 ± 0.2) and temperature (30 ^{o}C) were controlled. Anaerobic gas (N_2:CO_2=90:10) was flushed through the medium at 5 l/h. The gas first went through titanium solution (81) to remove traces of oxygen, and then the gas output was connected to a water column, which kept the reactor at a slight overpressure in order to avoid possible leakage of oxygen into the vessel. The pipes for inlet and outlet gas, addition of medium and base/acid solution were stainless steel, or the butyl rubber tubings (Precision FDA Viton® Tubing, Cole-Parmer instrument Co., USA).

Media components were supplied separately to the fermentor at equal rates from two 10-liter bottles, to provide a final medium composition similar to the one used for batch enrichments, except for lower concentrations of acetate (0.125 mM), lactate (0.0625 mM), yeast extract (0.0001 %) and the electron-acceptor six-line ferrihydrite (1 mM). The first bottle contained filter-sterilized six-line ferrihydrite colloidal solution, at pH 4.0, while the second bottle contained all other medium components. Medium from each bottle was pumped into the fermentor at 18 ml/h (0.00225 mmol h^{-1} acetate, 0.001125 mmol h^{-1} lactate, 0.036 mmol h^{-1} six line ferrihydrite) in an alternate fashion: after each day of pumping, the pump was switched off for a day. The retentostat was inoculated from the most diluted batch enrichment of the Appels site, with F6L as electron acceptor, that was still positive (containing 10^{-5} diluted sediments). The reactor was run in retentostat mode for 16.8 volume changes of medium. Bacterial community profile and Fe (II) produced were monitored over time (see below). After stabilization of the bacterial communities, the fermentor was shifted to chemostat mode and run at a dilution rate of 0.02 h^{-1} for 9.1 volume changes.

Physiological characterization of selected enrichments and isolates.

To evaluate the potential for utilization of various electron acceptors, the above mentioned basal medium (41) was employed with one of the following electron acceptors,: NO_3^- (10

mM), SO_4^{2-} (10 mM), Fe (III) citrate (10 mM), amorphous ferrihydrite (10 mM)), lepidocrocite (α-FeOOH, 10 mM), goethite (γ-FeOOH, 10 mM), Fe (III) phosphate (prepared at a 1:1 PO_4/Fe (III) ratio, 10 mM), AQDS (5 mM), or sulfur (S^o) (20% vol/vol). These media were inoculated (3% vol/vol) with a fully grown culture of enrichments or isolates that had completely reduced Fe (III) citrate.

Consumption of glucose (3 mM), benzoate (0.5 mM), or H_2 was tested for in basal medium with 2.5 mM six-line ferrihydrite as the sole electron acceptor. H_2 was supplied by flushing the media for 5 minutes. A washed cell suspension was used as inoculum (2 % vol/vol).

The lower and upper temperature limits for growth were determined using basal medium containing Fe (III) citrate (10 mM), lactate (1 mM) and acetate (2 mM). Inoculated medium was incubated at 4, 12, 25, 30, 37, 42, 50 and 60 ^oC. pH tolerance for growth was tested using the same medium at pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Inoculated media were incubated at 30 ^oC in the dark, unless mention elsewhere.

Physiological characteristics were determined for selected isolates as well as for selected multi-species enrichments, after three transfers to fresh basal medium. For both research locations, two types of consortia were checked: a consortium prepared by mixing equal amounts of the four types of enrichments (each containing a different type of Fe (III), Fe (III) citrate, six-line ferrihydrite, hematite, or hematite plus AQDS) corresponding to the least diluted (10^{-1}) sediment samples, and a second consortium for which enrichments corresponding to the most diluted sediment samples that still were positive, were mixed. The mixed consortia were washed before inoculation. In addition, a consortia obtained from the retentostat at 16.8 volume change was used. This consortium revealed a stable bacterial community structure.

Cultivation-independent detection of specific iron reducers. DNA extraction from sediments was performed using the FastDNA spinkit for soil (Q.BIO-gene, USA). Cell suspensions from enrichments were used directly for PCR, i.e. without DNA extraction. Cell suspensions were first treated with an equal volume of oxalate solution (containing per liter 28 g ammonium oxalate monohydrate and 15 g oxalic acid) to dissolve iron precipitates (43). After centrifugation, the cell pellet was suspended in sterile water, which was subsequently used for PCR. DGGE (denaturing gradient gel electrophoresis) profiling after 16S rRNA-gene based PCR revealed that the profiles with cells used as template in PCR were indistinguishable from the profiles using DNA extracted from the cells (data not shown).

PCRs specific for iron-reducing genera and families were conducted: *Geobacteraceae* were targeted with primers 494f (22) and 825r (62). Sequences corresponding to *Shewanella* and *Geothrix* were amplified according to Snoeyenbos-West et al., and *Anaeromyxobacter* according to North et al. (51, 62)

Specific iron-reducers were quantified by performing PCR on serial 10-fold dilutions of DNA extracts. The proportion of specific iron reducers was estimated relative to the total number of *Bacteria.* For the quantification of total bacteria, primers were employed that amplified a part of the 16S rRNA gene corresponding to *E.coli* positions 357 to 518 (49).

DGGE profiling and statistical analysis. Microbial communities thus were analyzed by DGGE profiling of the amplified 16S rRNA gene fragment corresponding to *E. coli* positions 357 to 518, and subsequently subjected to cluster analysis as described previously (Chapter 3, 55). In order to determine the influence of different types of iron oxides (as electron acceptor) and dilution of sediments (as inoculum) on bacterial community structure in iron-reducing consortia, the similarity matrix was subjected to statistical analysis (non-parametric Kruskal-Wallis ANOVA tests), using Systat. 7.0, as described previously (72).

Phylogenetic analysis. 16S rRNA gene fragments corresponding to *E. coli* position 8 to 1512 were amplified from selected iron-reducing enrichments. Cloning, screening, sequencing and phylogenetic analysis of these amplicons was conducted as described previously (37, 55, Chapter 2, 3).

Analytical methods. Fe (II) was determined using the ferrozine assay (76). The concentrations of nitrate and nitrite were estimated using Merkoquant test strips (Merck GmbH, Germany). Sulfide was measured colorimetrically (65). The concentration of reduced 2,6-anthrahydroquinone disulfonate (AQDS) was determined spectrophotometrically at 450 nm wavelength (40).

RESULTS

Hydrogeochemistry of the research locations

Samples for hydrogeochemistry and microbiology were taken at two different times (February 2001 and November 2002). Previous observations (Hyacinthe, unpublished results) have shown that geochemical data follow seasonal trends, showing the same behavior within a particular season. At both locations, the concentrations of dissolved Fe (II) started to increase at 5 cm depth and were relatively high between sediment depths of 5 to 15 cm (Figure 2A, 2C). In addition, the ascorbate-extractable Fe (near neutral pH) at Appels was on average 75 μmol/g, approximately 2 times higher than that in Waarde. Its concentration did not change much up to 17 cm deep.

The concentration of dissolved sulfate and sulfur in pore water at the brackish Waarde site was nearly 30 times higher than at the freshwater Appels site, (Figure 2B, 2D). At Appels, the concentration of sulfate started to decrease from 12 cm depth downwards until it was nearly depleted at 19 cm depth (Figure 2B). At Waarde, a stable concentration

of sulfate was observed between 0 to 8 cm deep, further down there was a rapid decrease (Figure 2D). The sulfate concentrations started to decrease a larger depth (8-12 cm) than where the Fe (II) concentrations increased (from 5 cm). In contrast to sulfate, the concentration of sulfur was rather independent of depth at Appels (Figure 2B). At Waarde, the sulfur concentration profile is comparable to that of sulfate: high concentrations (5-6 mM) in the first 10 cm, then a rapid decrease downwards (Figure 2D). The pH was rather independent of depth, pH was 7.03 ± 0.1 (average ± standard deviation) for the Appels site while 7.71 ± 0.23 for the Waarde site.

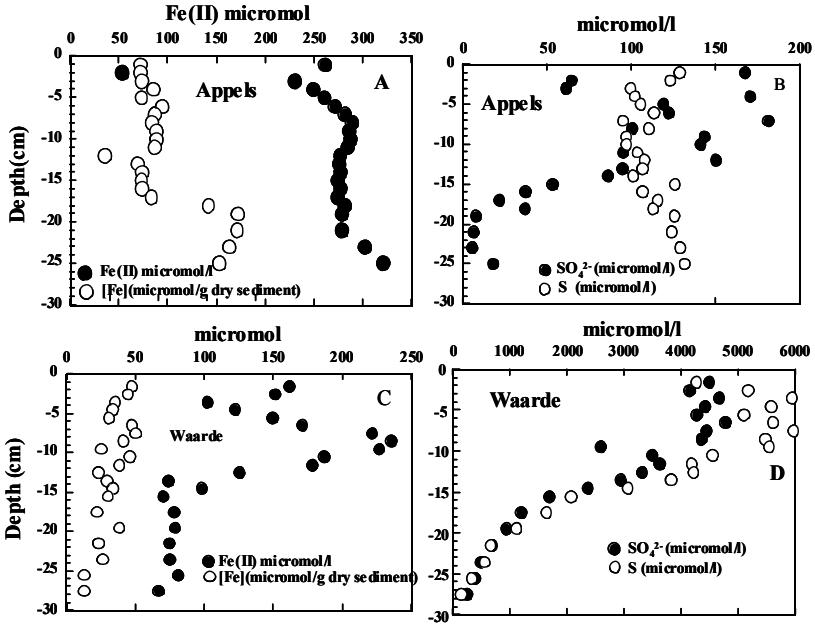

Figure 2. *Changes in hydrochemistry with depth at the Appels (A, B) and Waarde (C, D) locations in the Scheldt estuary. A, C, concentration of dissolved Fe (II) (●) and ascorbate extractable Fe (○) in pore water; B, D, concentration of dissolved SO_4^{2-} (●) and total S (○) in pore water. Measurements were performed in February 2001.*

Cultivation-independent analysis of Bacterial community structure and specific iron reducers

In order to compare the depth interval changes in hydrochemistry (Figure 2) to the variation in bacterial community structure, culture-independent DGGE profiling of amplified 16S rRNA genes was employed to sectioned sediment cores (Figure 3A). DGGE analysis revealed a complex microbial community structure, indicating a large bacterial diversity in both sites (Figure 3B). The bacteria community structure in freshwater sediments (Appels) was significantly ($p<0.05$) different from that in the brackish sediments (Waarde); the profiles revealed only 50% similarity between the sites. However, despite the changes in hydrochemistry (profiles of S and Fe) with depth (Figure 2), per location little correlation of

depth with the overall community structure was observed. The profiles clustered at a similarity of 95% for Appels and 85% for Waarde (Figure 3B).

Besides overall bacterial community structure, also the presence of four specific, well-known groups of iron-reducers was addressed by family- (*Geobacteracaea*) or genus-specific (*Anaeromyxobacter, Geothrix or Shewanella*) amplification of their 16S rRNA genes. *Shewanella*, *Geobacteraceae*, *Anaeromyobacter* and *Geothrix* were detected at all five intervals from 0 to 15 cm depth, at both Appels and Waarde. The relative contribution of these genera (or families) of iron reducers to the bacterial communities was determined for the mixed sediment sample, which was used for enrichments (corresponding to a depth of 5 to 15 cm; the depths at which Fe (II) concentrations had increased and were relatively high). Most-probable number PCR revealed that these 4 types of iron-reducers did not dominate the microbial communities at both sites. *Shewanella* was most abundant at approximately 1% of the total counts for both sites. Geobacters accounted for 0.01 % and 0.1% of the total bacterial population in Appels and Waarde sediments respectively, while *Geothrix* and *Anaeromyxobacter* contributed only 0.001%.

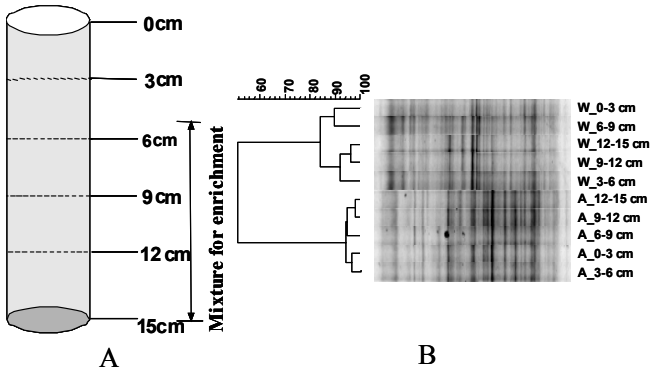

Figure 3. A*: Scheme showing how sediment cores from Appels and Waarde sites were subsampled for cultivation-independent DGGE analysis of their microbial community structure. Five core-slices were prepared, corresponding to depth segments of 0-3, 3-6, 6-9, 9-12 and 12-15 cm.* ***B****: UPGMA cluster analysis of DGGE profiles (30 to 55 % denaturant gradient, 4 hours at 200 V and 60 °C) of Bacteria in sediment samples obtained from several depths at the Appels and Waarde locations. 'A': sediment from Appels, 'W': sediment for Waarde.*

Community structure in iron-reducing enrichments

Iron-reducing enrichments of 10-fold serially diluted samples of sediment were established using four different types of Fe (III) [chelated Fe (III) oxide (Fe citrate), amorphous Fe oxide (ferrihydrite), crystalline Fe (III) oxide (hematite) and hematite plus humic acid analog (AQDS)] as sole electron acceptor (see M & M section). The number of culturable iron-reducing bacteria was 4.6 x 10^5 cells per gram sediment in Appels, nearly 20 times higher than in Waarde (2.4 x 10^4 per gram sediment).

Microbial communities of all iron-reducing enrichments were profiled by DGGE of amplified 16S rRNA gene fragments and analyzed numerically. UPGMA (unweighted pair group method with arithmatic mean) cluster analysis separated the samples into two

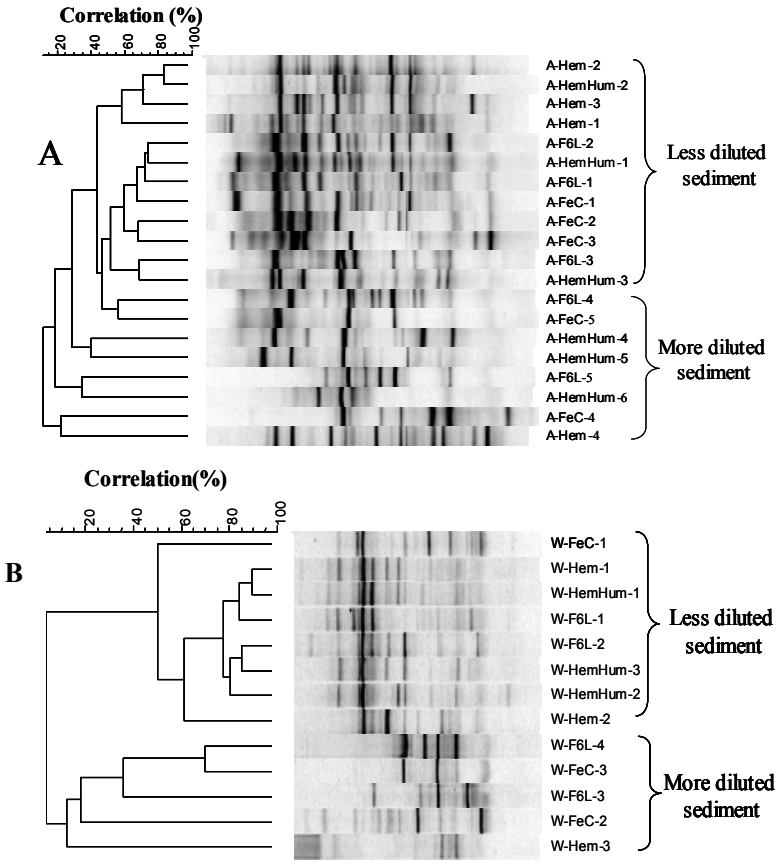

Figure 4. *UPGMA cluster analysis of DGGE profiles (30 to 55 % denaturant gradient) of the bacterial community in iron-reducing enrichments, containing different sources of Fe (III) as sole electron acceptor, and inoculated with sediment from Appels (A) or Waarde (B). 'A' and 'W' refer to Appels and Waarde, respectively; FeC stands for Fe (III) citrate, F6L for six line ferrihydrite, Hem for hematite, and HemHum for hematite plus the humic acid analog (AQDS). The following numbers after the character for each lane indicate the dilution factor of the inoculum, i.e. 5 for 10^{-5} times diluted sediment used as inoculum in the enrichment.*

major groups, both for Apples (Figure 4A) and Waarde enrichments (Figure 4B). Enrichments from the least diluted sediments (10^{-1} to 10^{-3} for Appels; 10^{-1} to 10^{-2} for Waarde) fell into one group. The other cluster contained enrichments from the more diluted sediments (10^{-4} to 10^{-6} for Appels; 10^{-3} to 10^{-4} for Waarde), with the exception of a few samples [enrichment with 6-line ferrihydrite at the dilution of 10^{-4} sediment (enrichment: A-F6L-4), enrichment with Fe citrate at dilution 10^{-5} (enrichment: A-FeC-5)

for Appels; enrichment containing hematite plus humic acid analog at dilution 10^{-3} (W-HemHum-3), and enrichment containing Fe citrate at dilution 10^{-2} (enrichment: W-FeC-2) for Waarde]. Non-parametric analysis of variance on the similarity matrix was performed in order to establish whether the dilution of the inoculum sediments and/or type of electron acceptor had a significant effect on the microbial community structure in the enrichments. In order to test the effect of dilution, the enrichment cultures were assigned to two groups based on the cluster analysis (Figure 4); for the Appels one group contained enrichments inoculated with 10^{-1} to 10^{-3} diluted sediments, the second group enrichments inoculated with 10^{-4} to 10^{-6} diluted sediments. For Waarde, enrichments inoculated with 10^{-1} to 10^{-2} diluted sediment were assigned to one group while the enrichments inoculated with 10^{-3} to 10^{-4} diluted sediment were assigned to the second group. A significant ($p<0.01$) effect of dilution on community structure was observed for both locations. When tested for the effect of electron acceptor, no significant ($p>0.05$) effect on microbial community structure was found. Therefore, we concluded that the applied dilution of sediment mainly determined community structure in the enrichments, not the type of electron acceptor. The lack of a significant effect of electron acceptor on community structure suggests the consortia are flexible in the use of the different types of Fe (III).

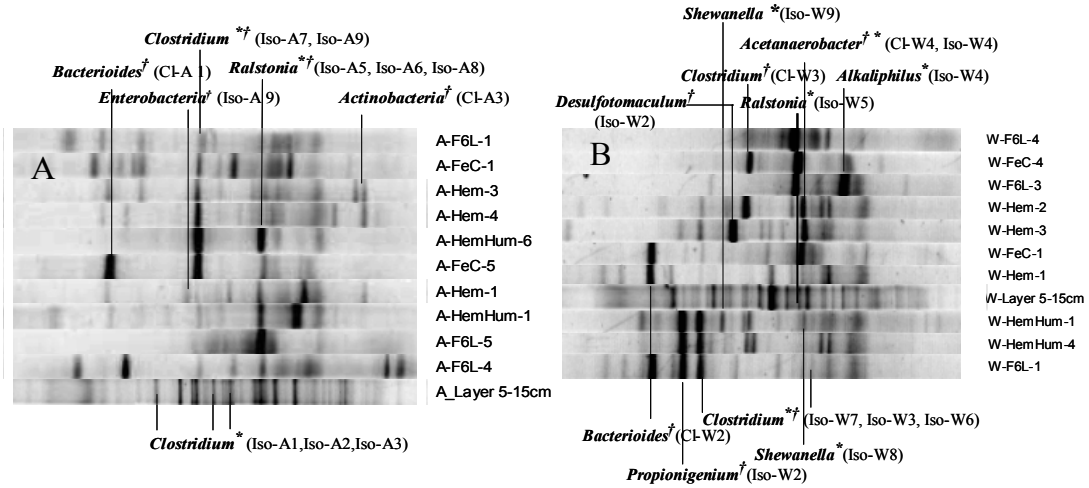

Figure 5. *16S rRNA gene PCR-DGGE based matching of individual isolates and clones, obtained after the 3rd transfer of iron-reducing enrichments, to the microbial fingerprint of the enrichment and sediment sample from which the enrichments were obtained.* ***A****. Appels;* ***B****. Waarde. The environmental samples are indicated by 'A_layer 5-15 cm', or 'W_layer 5-15 cm'. Codes for the enrichments are explained in the legend of Figure 4, "*" refers to an isolate, "†" to a clone. The characters in the bracket behind the species names refer to Table 1.*

Strain isolation and phylogenetic analysis of iron-reducing enrichments. Enrichments inoculated with the least diluted sediments (10^{-1} dilution) as well as with the highest dilution that still revealed iron-reduction, were maintained by repeated transfer (at 1% v/v inoculum) to the same medium as used for the original enrichment. After three transfers,

the microbial community structure (i.e. the dominant bands in the DGGE profiles) had in general not changed significantly as compared to the original enrichments (data not shown). From a number of enrichments (Table 1), for which the DGGE analysis revealed intense bands that also occurred in DGGE profiles for other enrichments, the microbial community composition was determined in more detail by isolating nine pure cultures for each site and sequencing of their 16S rRNA genes (Table 1). These isolates were related to the community structure in the enrichments from which they were obtained, as well as to the sediments used for inoculation of the enrichments, by DGGE analysis. This analysis revealed that for most isolates their position in DGGE profiles corresponded to bands observed in the DGGE profiles of the enrichments and the sediments (Figure 5A, 5B). The isolates were also subjected to 16S rRNA gene-based phylogenetic analysis (Table 1, Figure 7). *Clostridium* and *Ralstonia* species appeared to be dominant, culturable microorganisms in most iron-reducing Appels enrichments, especially in enrichments inoculated with the most diluted sediment samples [Figure 5A, Table. 1; i.e. isolates Iso-A6 and Iso-A7 were retrieved from the enrichments F6L-5 and A-HemHum-6 at dilution 10^{-6} of inoculated sediment respectively]. The positions of their 16S rRNA gene fragments on DGGE also corresponded to two intense bands in the DGGE profiles from the sediment sample used to inoculate the enrichments (Figure 5A). Serial dilution on DNA extracted from the sediments, followed by 16S rRNA gene based PCR-DGGE revealed that these intense bands indeed corresponded to organisms that dominated the sediment numerically (data not shown).

Clostridium and *Ralstonia* were also dominant, culturable genera in enrichments from Waarde (Figure 5B, Table 1), as they were recovered from enrichments inoculated with the most diluted sediment samples, i.e. enrichment containing 6-line ferrihydrite at dilution 10^{-3}, 10^{-4} of inoculated sediment (enrichment: W-F6L-3, W-F6L-4), containing Fe (III) citrate at dilution of 10^{-4} (enrichment: W-FeC-4) for *Ralstonia* Iso-W5; while dominant *Clostridium* in the enrichments with hematite plus humic acid analog at dilution 10^{-4} (enrichment: W-HemHum-4) and with Fe (III) citrate at 10^{-4} (enrichment: W-FeC-4). When comparing DGGE bands corresponding to the isolates to sediments from which they were isolated, the position of the genus *Ralstonia* (Iso-W5) corresponded to an intense band observed for the sediment (lane W-layer-5-15cm in Figure5B). The position of *Ralstonia* (Iso-W5) on DGGE gel were identical to that of an iron reducer (isolate Iso-A8, Table 1) isolated from Appels (Figure 5A, Figure 7). Furthermore, isolates belonging to the genera *Alkaliphilus* and *Shewanella* were obtained from Waarde enrichments, although only for the least diluted enrichments (10^{-1} to 10^{-3}).

The microorganisms giving rise to the most dominant bands in DGGE in some enrichments could not always be obtained as pure cultures, therefore also clone-libraries were constructed. The clones representive of more intensive bands corresponding to the enrichments in which clones were retrieved were subjected to sequencing and phylogenetic

Table 1. Identities and origin of clones and isolates obtained from iron-reducing batch enrichments inoculated with either Appels ('A') or Waarde ('W') sediments*.

Code	Origin	Closest relative in Genebank	Accession no.	Similarity (%)	Phylum (class)
Appels clone					
Cl-A1	A-FeC-5	Uncultured bacterium clonePL-7B7	AY570639	98	Bacteroidetes
Cl-A2	A-Hem-1	*Escherichia colis* 0157	AP002566	99	Proteobacteria (γ)
Cl-A3	A-Hem-1	Uncultured bacterium SJA-181	AJ009505	97	Actinobacteria
Appels isolate					
Iso-A1	sediment	*Clostridium* sp	Y10030	98	Firmicutes
Iso-A2	sediment	*Clostridium saccharolyticum*	Y18185	98	Firmicutes
Iso-A3	sediment	*Clostridium* sp CITR8	AY221993	99	Firmicutes
Iso-A4	sediment	*Enterobacter amnigenus*	AB004749	99	Proteobacteria (γ)
Iso-A5	A-HemHum-6	*Ralstonia* sp	AY177368	99	Proteobacteria (β)
Iso-A6	A-F6L-5	*Ralstonia* sp	AY177368	99	Proteobacteria (β)
Iso-A7	A-HemHum-6*	*Clostridium* sp 13A1	AY554421	97	Firmicutes
Iso-A8	A-FeC-5	*Ralstonia* sp 50	AY177368	99	Proteobacteria (β)
Iso-A9	A-FeC-5	Unidentified *Clostridiaceae* pDH-A	U85415	95	Firmicutes
Waarde clone					
Cl-W1	W-F6L-1	*Propionigenium maris*	Y16800	98	Fusobacteria
Cl-W2	W-F6L-1	Uncultured bacterium mlel-2	AF280841	98	Bacteroidetes
Cl-W3	W-FeC-4	*Clostridium subterminale* isolate DSM6970	AF241844	99	Firmicutes
Cl-W4	W-FeC-4	*Acetanaerobacter thermtolerans*	AF358114	99	Firmicutes
Waarde Isolate					
Iso-W1	W-F6L-3	*Alkaliphilus crotonoxidans*	AF467248	96	Firmicutes
Iso-W2	W-Hem-3	*Desulfotomaculum guttoideum*	Y11568	99	Firmicutes
Iso-W3	W-HemHum-4	*Clostridium sp* CITR8	AY221993	99	Firmicutes
Iso-W4	W-FeC-4	*Acetanaerobacter thermotolerance*	AF358114	98	Firmicutes
Iso-W5	W-F6L-4	*Ralstonia* sp	AY177368	99	Proteobacteria (β)
Iso-W6	W-HemHum-1	*Clostridium glycolicum*	AY007244	98	Firmicutes
Iso-W7	W-F6L-1	*Clostridium* sp CITR8	AY221993	99	Firmicutes
Iso-W8	W-HemHum-1	*Shewanella* sp MK03	AY690713	99	Proteobacteria (γ)
Iso-W9	W-HemHum-2	*Shwanella* sp A6 mk	AF319767	98	Proteobacteria (γ)

** In the column 'Code', 'Cl' stands for clone, 'Iso' for isolate and the following digital numbers are different isolates or clones. In the column denoted 'Origin', 'FeC' represents Fe citrate; Hem: hematite; HemHum: hematite plus humic acid analog; F6L: 6 line ferrihydrite and the following digital number is the dilution factor (i.e, 1 for 10^{-1}, containing 0.1g sediment). '*': the 2nd subculture. Similarity (%) refers to the similarity of the cloned sequence to the closest relative, which is mentioned in column 3.*

analysis. Sequences belonging to *Bacteroidetes* (in Appels and Waarde enrichments), *Actinobacteria* (Appels), *Enterobacteria* (Appels), *Acetanaerobacter* (Waarde), and *Propionigenium* (Waarde) were encountered (Figure 5A, 5B). Some species, such as *Bacteroides hypermegas, Escherichia coli,* and *Cellulomonas sp*., closely related to these sequences, have been reported to be capable of dissimilatory Fe (III) reduction (39). Therefore we also expect that species from the groups mentioned above may contribute to iron reduction. The presence of these various phylotypes indicates a large diversity in iron reducers in the Scheldt estuary.

Cultivation-independent analysis with family- or genus-specific primers revealed that the well-known iron-reducers *Shewanella, Geobacter, Anaeromyobacteria* and *Geothrix* were only detectable in enrichments containing the least diluted sediment, i.e. up to a dilution of 10^{-2}.

Table 2. Identities and origin of clones obtained from iron-reducing retentostat enrichments inoculated with Appels ('A')*.

Code	Closest relative in Genebank	Accession no.	Similarity %	Phylum (class)
Cl-A4	uncultured bacterium clone KD3-5	AY188305	97	Bacteroidetes
Cl-A5	Uncultured *Ralstonia sp.* clone EUB40	AY693819	99	Proteobacteria (β)
Cl-A6	*Ralstonia sp.* 1F2	AY509958	99	Proteobacteria (β)
Cl-A7	uncultured bacterium clone KD3-5	AY188305	97	Bacteroidetes
Cl-A8	*Bacillus fusiformis*	AJ310083	99	Firmicutes
Cl-A9	uncultured bacterium clone KD3-5	AY188305	97	Bacteroidetes
Cl-A10	Uncultured *Ralstonia sp.* clone EUB40	AY693819	99	Proteobacteria (β)
Cl-A11	uncultured *Pietermartzburg bacterium* Y14-3	AF312217	97	Firmicutes
Cl-A12	*Bacteriodes sp* 253c	AY082449	99	Bacteroidetes

**'Cl': clone; the following digital numbers indicate different clones. The clones were retrieved from the retentostat culture with stable microbial community (16.8 volume change).*

(Semi-) continuous retentostat enrichment

Batch enrichments especially enrich for fast growing species. In order to enrich more selectively for slow-growing iron-reducers, semi-continuous cultivation in a fermentor with biomass retention (retentostat) was conducted. Changes in microbial community were monitored during the cultivation period of nearly one year by DGGE profiling of the amplified 16S rRNA gene as well as by measuring Fe (II) production. During the first 140 days of retentostat culturing (16.8 volume changes), the number of DGGE bands in profiles increased and then stabilized (Figure 6, data not shown). After stabilization of the community profile, the fermentor was shifted to chemostat mode (continuous culturing without biomass retention) and maintained at a dilution rate of 0.02 h^{-1}. During chemostat operation, the complexity of the community profiles decreased. After 38 days of chemostat

cultivation (9.1 volume changes), the microbial community structure became stable and did not change during subsequent half a year of cultivation, i.e. 42.7 additional volume changes) (Figure 6).

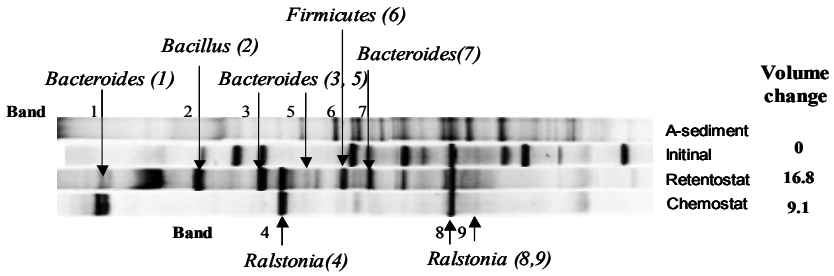

Figure 6. *16S rRNA gene PCR-DGGE based matching of individual clones to the microbial fingerprint of a batch enrichment used to inoculate a retentostat enrichment experiment ('initial'), the retentostat after 140 days (16.8 volume change) and the chemostat 38 days (9.1 volume change) after it was switched from retentostat to chemostat (on day 38 after starting the retentostat), as compared to the original sediment sample that was used as inoculum. The numbers behind the species names refer to Table 2.*

Sequencing of 16S rRNA gene fragments revealed that microorganisms belonging to *Bacteroides* (band 1, 3 and 5 in Figure 6; Table 2, and Figure 7) and *Ralstonia* (band 4, 8 and 9), and to a lesser extent to uncultured *Firmicutes* (band 6) and *Bacillus* (band 2) accumulated in the fermentor. Band 8 (*Ralstonia)* corresponded to an intense DGGE band observed for the environmental sample (Figure 5A), and the sequence of *Ralstonia*-like phylotype was identical to isolate Iso-A8 obtained from batch enrichments (Figure 7). On the other hand, some species-specific DGGE bands [i.e. band 3 and 7 (*Bacteroides*)], and band 6 (Uncultured *Firmicutes*) disappeared during chemostat culturing. Clones and isolates recovered from batch and retentostat enrichments were affiliated to five phyla (Table 1, 2, Figure 7), *Firmicutes* and *Proteobacteria* corresponding to68% of phylotypes for Appels and 73% for Waarde. Species affiliated to *Bacteroidetes* corresponded to 22% (Appels) and 20% (Waarde) of the phylotpyes. A small proportion of the sequences corresponded to *Fusobacteria* (6.7%, Waarde) and *Actinobacteria* (4.6%, Appels).

Physiological characterization. To evaluate and compare the physiological ability to (i) use alternative electron acceptors and donors, (ii) grow at different temperatures and (iii) tolerate non-neutral pH, consortia prepared from selected batch enrichments (as described in the M & M section), a consortium from the 140 day-old retentostat (16.8 fold volume change), and some isolates which dominated in sediment, were selected.

As shown in Table 3, consortia and isolates from both sites were able to reduce various types of Fe (III), including the predominantly bioavailable iron (III) phosphate. AQDS reduction was more often observed for Waarde than for Appels; whereas the humic-

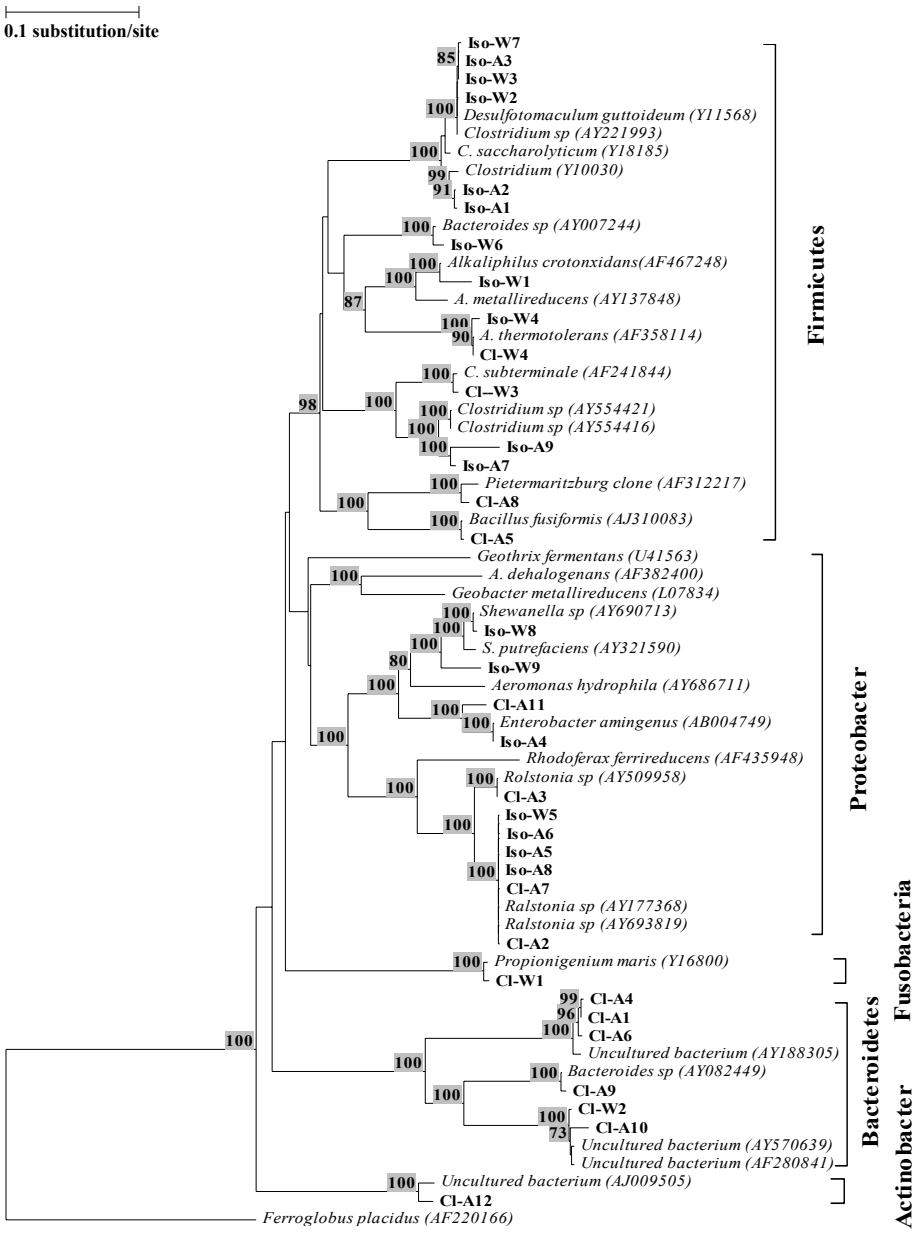

Figure 7. *Phylogenetic tree based on almost complete 16S rRNA sequences for isolates and clones obtained from the iron-reducing enrichments containing freshwater sediments (Appels) or brackish water sediment (Waarde). A neighbor-joining analysis with Jukes-and-Cantor correction was performed on unambiguous base positions. 1360 base pairs were taken into account for phylogenetic tree construction. Only Bootstrap values above 70% are shown. Codes and origin of the clones and isolates, shown in bold-face in the tree, are explained in Table 1 and 2.*

acid analog AQDS was only reduced by the least diluted Appels sediment. Sulfur reduction was observed in all cases for Waarde, but not for any of the tested isolates from Appels. Only consortia could conduct sulfate reduction. Consortia from Appels (both the consortia corresponding to the least and to the most diluted sediment samples) and Waarde (least diluted) could not reduce nitrate while the other consortia and, remarkably, isolates recovered from the non-denitrifying consortia, could (Table 3). All tested cultures except Iso-A8 (*Ralstonia*) and Iso-W1 (*Alkaphilius*) were able to carry out ferrihydrite reduction coupled to the oxidation of glucose. Also, all tested cultures were capable of performing dissimilatory Fe (III) reduction using H_2 as electron donor. Benzoate was used by all consortia and isolates Iso-W1 (*Alkaphilius*) and Iso-W9 (*Shewanella*) (Table 3).

Waarde consortia containing the least diluted sediments and the *Alkaliphilus*-like isolate were able to conduct iron reduction in the temperature range from 4 to 50 ^{o}C. The fastest iron-reduction occurred between 25 and 30 ^{o}C. A relatively high tolerance to pH (growth at 5.0 to 9.0) was observed for most isolates and consortia.

Table 3. Utilization of electron acceptors and donors, as well as temperature range for growth and pH tolerances for selected iron-reducing consortia and isolates.

	Electron acceptors									Electron donors			Temp	pH
Sample	Feci	Ferri	Lepid	Geothite	Fe-P	AQDS	Sulfate	Sulfur	Nitrate	Glucose	Benzoate	H_2	range	range
Apples														
least*	+	+	+	+	+	+	+	+	-	+	+	+	4-42^{o}C	5-9
most*	+	+	+	+	+	-	+	+	-	+	+	+	4-42^{o}C	5-9
fermentor*	+	+	+	+	+	-	+	-	+	+	+	+	4-37^{o}C	5-8
Iso-A8	+	+	+	+	+	-	-	-	+	-	-	+	4-30^{o}C	6-8
Waarde														
least*	+	+	+	+	+	+	+	+	-	+	+	+	4-50^{o}C	5-9
most*	+	+	+	+	+	-	+	+	+	+	+	+	4-42^{o}C	5-9
Iso-W1	+	+	+	+	+	+	-	+	+	-	+	+	4-42^{o}C	5-9
Iso-W6	+	+	+	+	+	+	-	+	+	+	-	+	4-42^{o}C	5-9
Iso-W7	+	+	+	+	+	+	-	+	+	+	-	+	4-50^{o}C	5-9
Iso-W9	+	+	+	+	+	+	-	+	+	+	+	+	4-37^{o}C	6-8

"": consortia. Codes 'least' refers to the iron-reducing consortium resulting from mixing enrichments grown with different electron-acceptors containing least diluted sediments and inoculated with 10^{-1} diluted sediment; 'most' refers to the iron-reducing consortium resulting from mixing the positive enrichments corresponding to the most diluted sediment samples and to a different type of electron acceptor. 'fermentor' for consortium obtained from a retentostat enrichment with 140 day cultivation (16.8 volume changes). Codes for individual isolate refer to Table 1. "Fe-P: Fe phosphate", "+": utilized, "-": not utilized. 'Feci': Fe citrate; 'Ferri': Ferrihydrite; 'Lepid': Lepidocrocite; 'Temp': temperature.*

DISCUSSION

Iron reduction has been observed in marine (8, 20, 30) and freshwater sedimentary environments (13, 32, 70, 73). Especially in sedimentary environments where sulfide is produced, it is hard to distinguish between the abiotic and biotic components contributing to iron-reduction (67). In order to address the biological potential for iron-reduction, we combined field-scale geochemical measurements with laboratory experiments on the associated microbiology for a freshwater (Appels) and brackish (Waarde) location in the Scheldt estuary, Northwest Europe.

Hydrogeochemical evidence for enzymatic iron reduction in the Scheldt estuary

Field-scale measurements revealed the substantial presence of dissolved Fe (II) in pore water at both research locations. Fe (II) concentrations increased from 3 cm deep down to a depth of 8 cm and then stabilized for the freshwater Appels location, whereas it decreased for the sulfate-rich brackish Waarde site. Sulfide produced from sulfate or sulfur reduction can readily react with dissolved Fe (II), thus removing Fe (II) as FeS from pore water. Dissolved H_2S, a strong reductant contributing to abiotic Fe (III) reduction (32), was hardly detectable in pore water at both sites in a previous study (23). The concentration of sulfate did not decrease over 0-10 cm depth, suggesting little to no H_2S production from sulfate reduction, while an increase in Fe (II) was observed. This phenomenon suggests that the increase in Fe (II) probably is not due to indirect effects caused by sulfate-reduction, but rather results from microbe-mediated iron reduction. Abiotic iron reduction by other factors than sulfide, such as organic compounds from microbial fermentative metabolism, is negligible at the circumneutral pH in these locations (44).

Previous field-scale measurements indicated that ascorbate-extractable, bioavailable Fe (III) phosphate (25% Fe (III)-phosphate out of 77% ferric iron in the sediments determined by Mössbauer analyses) (23) is abundant over the depths sampled here, even when sulfate-reduction is occurring. Iron-reducing microorganisms prefer amorphous Fe (III) over crystalline Fe (III) and the density of culturable iron-reducing *Bacteria* has been found to correlate positively with the concentration of ascorbate-extractable Fe (III) (45). Furthermore, Fe (III) phosphate is favored kinetically over amorphous iron hydroxides as a terminal electron acceptor by dissimilatory iron reducers which implies that Fe (III) phosphate is likely to be utilized by iron reducing bacteria *in situ* (23). All enrichments and isolates tested in our study were able to reduce Fe (III) phosphate.

On the basis of these geochemical analyses, we conclude that a high potential for enzymatic iron reduction is being expressed in the Scheldt estuary, especially in the upper 10 cm. Furthermore, factors affecting the rate of iron reduction, such as electron donor bioavailability and nutrients (nitrogen, phosphorous) are not likely to be a major limitation for the occurrence of enzymatic iron reduction since these substrates are continuously

supplied by the entrance of freshwater highly polluted by municipal, agricultural and industrial waste (79).

Iron reducers associated with anaerobic shallow sediments in the Scheldt estuary

The field-scale hydrochemical analysis on the shallow sediments in the Scheldt estuary, which is strongly polluted with organic matter (75, 79) were complemented with characteristics of the associated iron-reducing microbial communities. The numbers of culturable iron reducers (4.6×10^5 and 2.4×10^4 cells g^{-1} sediment for Appels and Waarde, respectively) are comparable to those found in other shallow iron-reducing sediments (32, 45, 46), but in these earlier studies the microbial community composition was not determined in great details. The composition of iron-reducing communities has been investigated for other, mainly subsurface, environmental settings polluted with organic matter. Predominantly *Geobacter* species were observed in such circumneutral iron-reducing settings (2, 9, 26, 37, 55, 56, 62, Chapter 2, 3). Therefore it is surprising that species *Geobacteraceae* were not isolated in our present study and that cultivation-independent methods revealed that they constituted only a minor fraction of the microbial communities present. Also other well-known iron-reducers, such as *Anaeromyxobacter*, *Geothrix* and *Shewanella* were hardly recovered. On the other hand, our observation is in line with Lowe et al., describing that *Geobacter* and *Shewanella* were not predominant iron reducers in salt marsh sediments (45). Among the four types mentioned above, *Shewanella* was detected most often. *Shewanella* is a facultative iron reducer, also able to grow with other electron acceptors, and has been encountered in a variety of environments (74).

Instead of the well-known iron-reducers, facultative anaerobic *Ralstonia* and spore-forming *Clostridia* dominated microbial communities and enrichments. The *Ralstonia* isolates were able to grow with oxygen and other non-Fe (III) electron acceptors. The relatively high abundance of *Ralstonia* may possibly also relate to the presence of high concentrations of metals in the Scheldt estuary (82-85), which are up to 64 μg g^{-1} (sediment), As, 20 μg g^{-1} Cd, 0.20 mg g^{-1} Cr, 2.6 mg g^{-1} Cu, 3.3 μg g^{-1} Hg, 0.22 mg g^{-1} Ni, 0.46 mg g^{-1} Pb and 1.5 mg g^{-1} Zn (85). The presence of *Ralstonia* has also been reported for other environments heavily contaminated with toxic metal ions (16, 31, 47). *Ralstonia* is able to reduce selenite to elemental selenium (4, 57). However, the mechanism for reduction of selenite is largely unknown. This report is the first suggesting a role for *Ralstonia* in iron-reduction. *Ralstonia* did not use Fe (III) as a terminal electron acceptor associated with an electron transport chain, but rather as an electron-sink, i.e. to reoxidize NADH in substrate-level phosphorylation based energy metabolism (data not shown).

This was also the case for the second group of numerically important microorganisms in the Scheldt estuary, i.e. the strictly anaerobic, spore-forming *Clostridia*. Several iron-reducing *Clostridium* species have been described previously (10, 11, 17, 39, 53), all using Fe (III) as an electron sink, allowing them to harvest more free energy per unit carbon dissimilated. Iron-reducers that conserve free energy from electron transport

along an electron transport chain were less frequently isolated or observed using molecular methods than microorganisms using Fe (III) as a sink. One of these species belonged to the genus *Alkaliphilus sp.* and is potentially a novel species as it had only 96% similarity to *Alkaliphilus crotonoxidans.* Recently the first iron-reducing member of this genus, *Alkaliphilus metallireducens,* was described (80). Some of the characteristics of our isolate are quite distinctive from *A. metallireducens,* most notably its ability to use ferrihydrite, lepidocrocite, goethite, Fe phosphate, sulfur or nitrate as electron acceptor as well as its ability to grow at circumneutral pH and its higher temperature (50^{o}C) tolerance.

Propionigenium-like species dominated in some Waarde enrichments. Its closest relative (98 % similarity to *P. maris)* in Genbank originated from an estuarine sediment and was capable of fermenting carbohydrates to formate, acetate, ethanol and lactate when yeast extract was present (28). Thus, *Propionigenium* may not reduce Fe (III) but contribute to iron reduction for providing electron donors such as lactate or acetate, a role supported by its recovering from estuary environments rich in organic matter.

Bacteroides-like species were also predominantly present in iron-reducing enrichments and found in other iron-reducing environments (58, 68), and anaerobic landfills (14). Whether *Bacteroides* species are able to dissimilate Fe (III) is unknown. They probably function as fermentative bacteria to provide small molecular substrates, or lower the redox potential, which is necessary for iron reducers to function properly (64).

Overall, we observed a relatively large phylogenetic diversity in iron reducers in our study, certainly as compared to others (2, 9, 26, 37, 51, 55, 56, 62, 74, Chapter 2, 3). The dominating iron-reducers mainly used Fe (III) as an electron-sink and appeared quite versatile regarding changing environmental conditions, either by their facultative anaerobic growth with a range of electron-acceptors (*Shewanella, Ralstonia*) or by their ability to survive as spores (*Clostridia*). The relatively high abundance of these facultative iron-reducers, as well as the rather stable community structure over depth and the observation of *Shewanella*, *Anaeromyxobacter, Geothrix* and *Geobacter* at all tested depths, may relate to a combination of the following factors. They are (i) high microbial activity due to organic pollution (5, 79), leading to depletion of oxygen in the upper 0.3-0.5 cm of the sediments and denitrification in the upper 0.6 cm (23, 36), making it possible for iron reducers to survive and function even in shallow sediments; (ii) mechanical forces such as wind and tides, as well as bioturbation and bioirrigation by macrofauna (nematodes and polychaetes) continuously rework the sediment and redistribute its associated microorganisms and nutrients over depth (33). This should cause continuously changing environmental conditions, especially with respect to concentrations of electron acceptors, and may require that the microorganisms are flexible in their use of electron acceptors.

Evaluation of the enrichment strategies employed

Although 16S rRNA sequence analysis has many advantages over cultivation, and yields considerable information on microbial communities, culture-dependent methods are still

indispensable in understanding the physiological traits and improve knowledge on the functional roles played by particular species. Microorganisms at subsurface usually grow at very low substrate concentrations and growth rates, with doubling times ranging from days to years (77). Therefore simple batch enrichments tend to select for the fastest growing microorganisms and may fail to enrich for the numerically dominant species (3). Therefore, we employed dilution-to-extinction enrichments to allow the numerically dominant perhaps slowly growing microbes from the sediments to be enriched and isolated, and to select against rare but opportunistic and fast-growing species (7, 78). Previously this approach was employed successfully to enrich and isolate microorganisms selectively from complex microbial communities. This pertained to the numerically dominant and culturable naphthalene-degrading sulfate reducers (18), to methanotrophic bacteria (78) and to numerically important bacteria from a marine enrichment community (15). By combining the enrichments with molecular analysis we were able to show that the applied dilution-to-extinction enrichment indeed strongly affected community structure in the enrichments. Also the *Ralstonia* and *Clostridia* species isolated from the most diluted, positive enrichments corresponded to relatively intense bands in cultivation-independent generated DGGE profiles of the environmental samples, from which these isolates were retrieved.

In addition, we applied retentostats to establish the enrichments. Retentostats, continuous culturing devices with biomass retention, allow for a better simulation of environmental conditions (low substrate concentrations and growth rates less than 0.05 h^{-1}) (66, 71). As compared to batch enrichments, in retentostat-based enrichments the microbial community structure became more complex over time. Additional *Ralstonia*-type and *Bacteroides*-type species were retrieved by retentostat enrichment. Thus, retentostat enrichment appears to be a suitable and useful approach in enhancing the comprehensive understanding of the physiology of environmental microorganisms by the recovery of numerically important slowly-growing microbes that cannot easily be obtained in batch enrichments.

CONCLUSIONS

On the basis of our findings, we conclude that (i) a high potential for microbial iron reduction exists and is being expressed in the Scheldt estuary; (ii) *Ralstonia* and *Clostridium* are dominant iron reducers, whereas the well-known iron-reducers like *Shewanella, Geobacter, Anaeromyxobacteria, Geothrix,* and *Alkaliphilu* are not*;* (iii) using dilution-to-extinction batch enrichment and retentostat enrichment we were able to select for the numerically most dominant iron-reducers, which offer the possibility to assess their physiological capacities further with respect to the fluctuating environmental conditions they experience.

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Chapter 6

How *Geobacteraceae* may dominate subsurface biodegradation: physiological characteristics of *Geobacter metallireducens* in habitat-simulating retentostats

ABSTRACT

Geobacters dominate many iron-reducing subsurface environments and are associated with biodegradation. In order to enhance the understanding of the environmental role played by Geobacters, retentostat cultivation for *Geobacter metallireducens,* at growth rates simulating natural environments both under electron-acceptor and under electron-donor limitation was set up. Its maximum growth yield was between 0.03 and 0.1 C-mol biomass per C-mol acetate. The iron-reducing capacity was comparable to that of *Shewanella putrefaciens*, the highest initial iron reduction rate being observed for citrate-chelated Fe (III) (i.e. $7x10^{-9}$ $\mu mol^{-1}cell^{-1}$ h^{-1}), whilst the rates on amorphous ferrihydrite and crystalline nanohematite were 40 and 1500 times lower, respectively. The maintenance catabolism demand obtained in this study was much lower than the lowest reported for heterotrophic bacteria. We discuss how the extremely low maintenance energy demand and the ability readily to use alternative electron acceptors may enable Geobacters to be become ubiquitous, and dominant over other microorganisms with higher maintenance metabolism, in many iron-reducing subsurface settings.

In collaboration with Martin Braster, Hans V. Westerhoff and Wilfred F.M. Röling

INTRODUCTION

All cultured members of the family *Geobacteraceae* are capable of dissimilatory Fe (III) reduction. Geobacters are frequently found as predominant microorganisms in iron-reducing environments, especially in aquifers polluted by or enriched in organic matter (8, 9, 15, 19, 36, 40). The organic matter serves as electron donor, and from transfer of these electrons to Fe (III) free energy is harvested and then used to support growth (29). *Geobacter metallireducens* and *G. grbiciae* are the only iron-reducing species described to date that are capable of toluene oxidation (8, 28), while there are strong indications that *Geobacter spp*. are also involved in anaerobic benzene degradation (37). Therefore, Geobacters appear to be important in natural attenuation of organic compounds.

Physiological studies on environmentally important microorganisms are typically conducted in batch culture, where microbes grow at maximum growth rate and with high substrate concentrations. These conditions do not mimic the natural conditions well, where microorganisms usually grow at very low growth rates with doubling times ranging from days to years (52). Recently, an *in situ* doubling time of 15 days ($\mu = 0.0019\ h^{-1}$) was determined for an aquifer (31). Microbial physiology at low growth rates can differ quite considerable from the physiology at higher growth rates, for example due to the influence of growth rate on gene expression (18) or 'stringent regulation' (1, 47). Lower growth rates and substrate concentrations can be maintained with continuous culturing methods, such as chemostats and retentostats. Of these methods, chemostat cultivation is not suitable to study microbial physiology at rates lower than $0.05\ h^{-1}$, because of inhomogeneities in substrate concentrations caused by the low addition rate of medium (3, 43, 47). Therefore, the lesser known retentostat cultivation method is more suitable for studying the physiology of environmentally relevant microbes (42). During retentostat cultivation, fresh medium is supplied to the fermentor at a constant flow rate. Spent medium leaves the fermentor at a similar rate, while the biomass is retained by a filtration unit, resulting in the accumulation of biomass, therewith a continuous decrease in the rate of substrate supply per unit biomass, and hence a decrease in specific growth rate.

Monitoring and directing biodegradation could be greatly improved by further understanding of how the major groups of microorganisms involved in biodegradation, such as Geobacters, deal with the limitations imposed by their environment. Therefore, we studied the physiology of *G. metallireducens* under environmentally relevant conditions: *G. metallireducens* was grown in the retentostat under either electron-donor (acetate) limitation or electron acceptor (the humic acid analog 2,6-anthraquinone disulphonate (AQDS)) limitation. Maintenance energy demand (the catabolism required to keep cells alive at zero growth rate), maximum growth yield, the ability to use alternative electron acceptors simultaneously, and the iron-reducing capacity on different forms of Fe (III) oxides were determined.

We report that (i) using the retentostat *G. metallireducens* can indeed be grown in the laboratory at environmentally relevant conditions of extremely low supply of metabolic energy, and (ii) it has a remarkably low maintenance requirement, possibly explaining its dominance in its slow growth habitat.

MATERIALS AND METHODS

Organism and cultivation media. *Geobacter metallireducens* (DSM 7210) was purchased from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* GmbH (DSMZ), Germany. Strict anaerobic conditions were employed throughout the work. Cultures were maintained in modified mineral medium (30) consisting of (per liter) $NaHCO_3$: 2.5 g; NH_4Cl: 1.5 g; NaH_2PO_4: 0.6 g; KCl: 0.1 g; Na-acetate: 2 mM; and trace element solution (DSMZ medium 141), 10 ml, which was supplemented with 10 mM Fe (III) citrate as electron acceptor and kept under an anaerobic atmosphere (N_2:CO_2; 90:10). Bottles were sealed with butyl rubber stoppers and autoclaved at 121^{o}C for 15 min. 10 ml filter-sterilized vitamin solution (DSMZ medium 141) was added per liter medium after autoclaving and cooling. Maximum growth rate was determined in batch cultures containing mineral medium with 5 mM of the humic acid analog AQDS (anthraquinone 2,6-disulfonate, Sigma-Aldrich Co., Germany) or 10 mM Fe (III) citrate as sole electron acceptor. Inoculum for retentostat cultivation was prepared in mineral medium with 4.8 mM AQDS as electron acceptor. Media for retentostat cultivation had the same composition as the medium used for growing the inoculum, except for the concentrations of electron donor (acetate) and acceptor (AQDS). Medium for the AQDS-limited retentostat contained 2.0 mM acetate and 4.8 mM AQDS, for the acetate-limited retentostat 0.65 mM acetate and 4.8 mM AQDS. Medium for retentostat cultivation (20 liter) was prepared by autoclaving the medium without vitamins and carbonate solution, which were added after cooling. Next, the medium supply was made and kept anaerobic by flushing with anaerobic gas (N_2:CO_2=90:10). All incubations were performed at 30 ^{o}C.

Retentostat cultivation. The retentostat equipment was designed and built by the electronics and mechanics workshops of the Faculty of Earth and Life Sciences, Vrije Universiteit Amsterdam, The Netherlands, and has been described previously (38). A two-liter fermentor vessel with a working volume of 1.5 l was used. The culture volume was controlled by means of a liquid-level indicator and a retention unit (containing a 0.22 μm pore size filter), both inserted through the top plate of the fermentor. The liquid-level indicator regulated a peristaltic pump that kept the volume of the fermentor constant by withdrawing liquid from the culture through the retention unit. The culture was agitated by flat-blade, propeller-type impellers operating at 200 rpm. Culture pH (6.8±0.2) was controlled by the addition of 1 M HCl or NaOH, and the temperature was controlled at 30 ^{o} C. A gas mixture of N_2 and CO_2 (90:10) was led through the culture at 1.3 l/h. Traces of

oxygen in the gas mixture were removed by leading the gas first through a strongly reducing titanium (III) citrate solution (54). The gas outlet was connected to a water-filled column, which kept the fermentor at a slight overpressure, in order to avoid possible leakage of oxygen into the fermentor. The pipes for the inlet and outlet of gas and for the addition of medium and base/acid solutions were made from stainless steel. Tubing consisted of Precision FDA Viton® Tubing (Cole-Parmer, USA). Medium was added to the fermentor by a peristaltic pump (easy-load II model 77202-60, Cole-Parmer Instrument Company, US). A supply rate of 0.17 mmol h^{-1} AQDS was maintained for the AQDS-limiting retentostat, a supply rate of 0.020 mmol h^{-1} acetate for the acetate-limiting retentostat. The fermentor and medium reservoir were kept in the dark.

Retentostat experiments were initiated by adding 80 ml inoculum to 1.5 liter anaerobic medium in the fermentor through a sampling port. The fermentor was first operated in batch mode for a week. When nearly all AQDS had been reduced (in the case of the AQDS-limiting retentostat) or when nearly all acetate had been consumed (for the acetate-limiting retentostat), the fermentor was switched to retentostat mode by starting the medium supply as well as the withdrawal of excess liquid through the retention unit.

Analytical measurements. Samples for measurements on cells were drawn anaerobically from the fermentor. Cell numbers and sizes were measured immediately using a Coulter Multisizer II (Coulter Electronics, Inc., England). Protein content was determined with the NanoOrange Protein Quantitation Kit (Molecular Probes, Inc. USA). Dry weight was measured as previously described (47). Total organic carbon (TOC) was determined by a Total Carbon Analyzer, Type DC-190 (Rosemount Analytical Inc., CA, USA) (46). Live and dead staining (Bacterial Viability Kit, Molecular Probes Inc., USA) was used to check whether dead cells accumulated in the fermentor over time by confocal laser scanning microscopy (Bio-Rad Radiance 2000, USA).

Cell-free filtrate was collected anaerobically into 10 ml serum bottles. In order to quantify the amount of reduced AQDS (AH_2QDS), the filtrate was mixed with anoxic 10 mM Fe (III) NTA solution within an anaerobic chamber (PLAS-LABS, USA). Fe (II) produced from abiotic Fe (III) reduction by AH_2QDS was measured by the ferrozine assay (50). The concentration of AH_2QDS was calculated on the basis of 2 Fe (II) produced per AH_2QDS. Acetate was measured by High-Performance Liquid Chromatography (Shimadzu, Japan) on an Aminex HPX87H column (Biorad), after treating the filtrate with 35% PCA and 7 M KOH to remove the proteins.

Determination of the simultaneous expression of alternative redox pathways. The ability to use alternative electron acceptors, without the requirement for gene induction, was tested in the presence of 150 μg/ml of the protein-synthesis inhibitor chloramphenicol. At the end of the experiments, cells were harvested anaerobically from the fermentor and washed 2 times with an excess of anaerobic washing medium. The washing medium was

nearly identical to the cultivation medium but lacked the electron acceptor (for cells from the AQDS-limiting retentostat) or electron donor and acceptor (for cells from the acetate-limiting retentostat). The final cell concentration in the test media was approximately the same as that in the fermentor from which the washed cell suspension was prepared. Carry-over of AQDS from the cultivation medium to the test media via the washed cell suspension was ruled out: no immediate Fe (II) production was observed after mixing of a small aliquot of the washed cell suspension with an equal aliquot of 10 mM Fe (III) NTA. The test media had the same chemical composition as the medium used for retentostat cultivation except for the electron acceptor, either nitrate (10 mM), Fe (III) citrate (10 mM) or six-line ferrihydrite (2 mM), and the presence of 150 µg/ml chloramphenicol. Six-line ferrihydrite was supplied as colloidal solution, which had been prepared as described (39). Production of AH_2QDS (measured as described above), Fe (II) (50) and nitrite (Merkoquant test strips, Merck, Germany) was determined after one week of incubation at 30 ^{o}C.

Capacity for iron reduction. The capacity to reduce different forms of Fe (III) was determined in media containing either 40 mM Fe (III) citrate, 40 mM ferrihydrite or 40mM hematite, according to Bonneville *et al.* (2). Ferrihydrite and hematite were prepared as described by Lovley (25) and Schwertmann (39), respectively. The electron acceptor concentrations were saturating (2). The capacity test media were inoculated with the same cell suspension as described in the previous section. The test media were regularly sampled during a period of three days. Samples were stored in a final concentration of 0.5 M HCl. Before measuring Fe (II), samples were placed overnight at 60 ^{o}C in order to extract Fe (II) efficiently from the precipitates. The amount of Fe (II) was determined with the ferrozine assay (50). Initial iron reduction rates were calculated in µmol $cell^{-1}$ h^{-1}.

Determination of maintenance energy demand and maximum growth yield. For retentostats, maintenance energy demand and maximum growth yield can be estimated by fitting the following equation to the biomass data (47):

$$x(t) = \frac{r_S}{m_S} - \left(\frac{r_S}{m_S} - x_0 \right) \cdot e^{-m_S \cdot Y_{xSm} \cdot t} \qquad [1]$$

In which t = time (h), $x(t)$ = biomass (biomass units) as a function of time t, r_S = addition rate of the growth-limiting substrate (mmol/h), m_S = maintenance energy flow (mmol/(biomass unit * h)) and Y_{xsm} = maximum growth yield (biomass units/mmol of limiting substrate). As wall-growth was frequently observed during retention culturing, the biomass data could not always be used to obtain m_s and Y_{xsm} via equation 1. Therefore, an

alternative approach was applied, i.e. fitting measured biomass production rates as a function of time t ($r_x(t)$) to the time derivative of equation 1:

$$r_x(t) \equiv \frac{dx(t)}{dt} = m_s \cdot Y_{xsm} \cdot \left(\frac{r_S}{m_S} - x_0 \right) \cdot e^{-m_s \cdot Y_{xsm} \cdot t} \qquad [2]$$

The biomass production was calculated based on acetate utilization. Acetate is used in biomass formation and respiration. The stoichiometries of the respiration of acetate with AQDS as two-electron acceptor are given by the following reaction equation:

$$CH_3COO^- + 4\ AQDS + 4\ H_2O \rightarrow 4AH_2QDS + 2\ HCO_3^- + H^+ \qquad [3]$$

It was assumed that all carbon in acetate that is not used for respiration, ended up in biomass (4, 14). Then, the rate at which acetate is incorporated in new biomass is:

$$r_{[Acetate]x}(t) = 2 \cdot f \cdot ([Acetate]_{reservoir} - [Acetate]_{filtrate}(t) - 0.25 \cdot [AH_2QDS]_{filtrate}(t)) \qquad [4]$$

$r_{[acetate]x}$ is in C-mol biomass per hour, f equals the flow- or pump-rate (l/h), $[Acetate]_{reservoir}$ is the acetate concentration in the medium supply, while $[Acetate]_{filtrate}(t)$ and $[AH_2QDS]_{filtrate}(t)$ are the concentrations in the filtrate, at time t. Finally, m_s and Y_{xsm} were obtained by fitting the calculated r_x, at different time points (eq. 4) to equation 2. Fitting was done in Kaleidagraph 3.09 (Synergy Software, USA).

RESULTS

Retentostat cultivation of *G. metallireducens* with AQDS as electron acceptor

Acetate is a major fermentative intermediate and electron donor for iron-reducing organisms in anoxic environments (23), where it can be a growth-limiting substrate due to low fermenting activity or preferential utilization by nitrate-reducing or Mn (IV)-reducing microorganisms (24). Accordingly we used acetate as electron donor.

Most iron-reducing microorganisms, including *Geobacter* species, are capable of using Fe (III) citrate as electron acceptor (7, 29). Initial experiments in which Fe (III) citrate was employed as electron acceptor were not successful due to the formation of Fe (II) precipitates and subsequent blockage of the filter membrane draining liquid from the reactor. Therefore, the humic acid analog AQDS was selected as an alternative electron acceptor for retentostat cultivation. AQDS is well-soluble and *G. metallireducens* grew well under conditions where AQDS was the sole electron acceptor. Its maximum growth rate (μ_{max}) on AQDS was 0.16–0.17 h^{-1} while on Fe (III) citrate it was 0.09-0.11 h^{-1}. Next,

retentostat cultivation was performed under electron acceptor (AQDS) limitation or electron donor (acetate) limitation.

In the AQDS-limited retentostat, AQDS was indeed completely reduced; the concentration of reduced AQDS was approximately equal to the amount of AQDS supplied in the medium (4.8 mM), throughout the experiment (Figure 1A). The concentration of residual acetate was also rather constant (0.76 mM on average). HPLC analysis did not reveal other organic compounds in the filtrate, neither did it indicate that AQDS was being degraded. Biomass, expressed in terms of number of cells in the reactor, increased almost linearly with time during the experiment (Figure 1C). A similar observation was made for the protein content of the fermentor (data not shown). At the end of the experiment about 13% of the biomass was attached to the walls of the fermentor (Figure 1C). Therefore, maintenance energy demand and maximal growth yield were not estimated by fitting the suspended biomass data to equation 1 in Material and Methods, but by fitting the rate of accumulation of acetate in biomass to equation 2. The rate of biomass production was calculated for each measuring point on the basis of the residual acetate concentration, the acetate concentration in the medium supply and the amount of reduced AQDS, according to equation 4 in Material and Methods (Figure 1B).

As suggested by the linearity of biomass accumulation with time, maintenance catabolism demand was statistically insignificant, for *G. metallireducens* growing under AQDS-limitation (Table 1). The maximum growth yield was 0.04-0.06 C-mol biomass/mol AQDS or 0.075-0.10 C-mol biomass/C-mol acetate. Based on the measured protein contents, total organic carbon and dry weight (data not shown), we determined that 1 C-mol biomass equaled 7.2 x 10^{13} cells. Therefore, the yield can also be expressed in cell numbers, 1.4-1.8 x 10^{12} per mol electron. The protein content of the cells at the end of the experiment was 51 % of dry weight. Although the experimental biomass data themselves were not used for the estimation of maintenance energy demand and maximum growth yield, the fitted biomass equation 1 (with the fit parameters in Table 1) corresponded very well to the experimental data, especially if wall growth was taken into account (Figure 1C).

In the acetate-limited retentostat, acetate was completely consumed (< 0.01 mM; Figure 2A) while the amount of reduced AQDS was around 2.4 mM, decreasing slightly during the experiment (Figure 2A). HPLC analysis did not reveal the presence of other organic compounds in the filtrate, nor did it indicate that AQDS was being degraded. The cell numbers as measured in samples taken from the fermentor appeared to decrease slightly during this experiment. There was wall-growth however; at the end of the experiment with 55 % of the cells were attached to the surfaces of the fermentor (Figure 2C). Therefore, also in this case maintenance catabolism was determined by fitting the rate of accumulation of acetate in biomass (Figure 2B).

Maintenance catabolism demand was again statistically insignificant (Table 1). Maximum growth yield was between 0.03 and 0.08 C-mol biomass/C-mol acetate (Table 1). From the measured protein contents, total organic carbon and dry weight (data not

shown), it was calculated that 1 C-mol biomass equaled 5.8 x 10^{13} cells. The yield expressed in terms of number of cells produced per mole of electron was 0.4-1.2 x 10^{12}. Proteins constituted 42% of the dry weight of the cells at the end of the experiment. The fit of the biomass to equation 1 (for the estimated parameters cf. Table 1), corresponded reasonable well to observed initial and final biomass in the fermentor, provided that wall growth was taken into account (Figure 1B): The fitted biomass at the end of the experiment was 15 % higher than that observed experimentally.

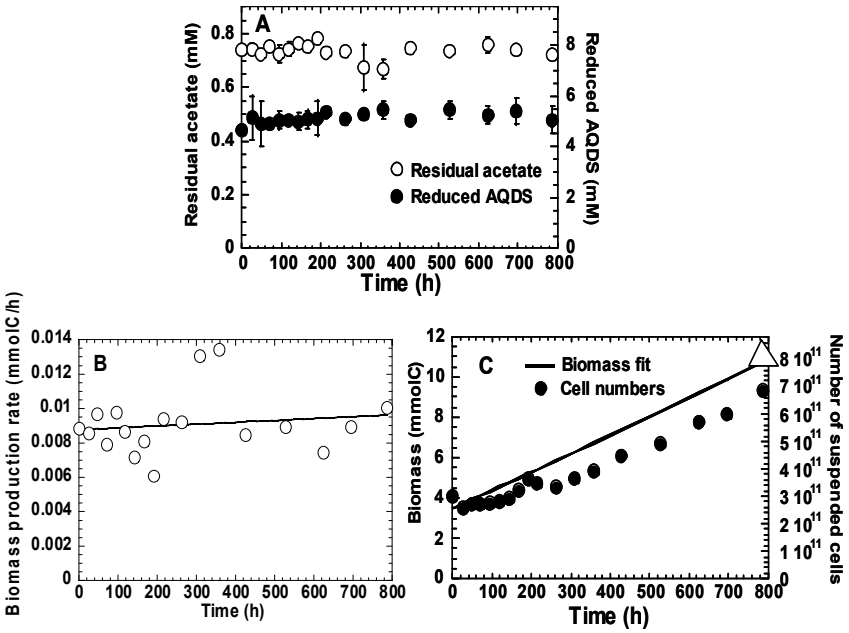

***Figure 1**. Substrate turnover and biomass data for G. metallireducens grown in continuous culture with 100 % biomass retention under AQDS-limiting conditions. **A**: concentrations of reduced AQDS (●) and residual acetate (○). Bars indicate standard deviation (n=2); **B**: biomass production rate (r_x_t) over time (expressed as mmol C_1-acetate h^{-1}). The line indicates the result of fitting equation 4 to the calculated production rates; **C**: comparison of measured cell numbers in the culture (●) to the fitted biomass (—), based on equation 1 and fitted growth parameters (Table 1), over time. (Δ) indicates the biomass after scrapping cells from surfaces in the fermentor.*

In both experiments, cell size increased over time, shifting from a diameter of 0.70 μm at the start of the experiment to 0.75 μm at the end (Figure 3A and 3B). This observation differs from those made on *Nitrosomonas europaea* (42, 43) and *Nitrobacter winogradskyi* (42), the cell sizes of which decreased during retentostat cultivation. The cells were alive; no dead cells were observed after Live/Dead staining at the end of the experiment.

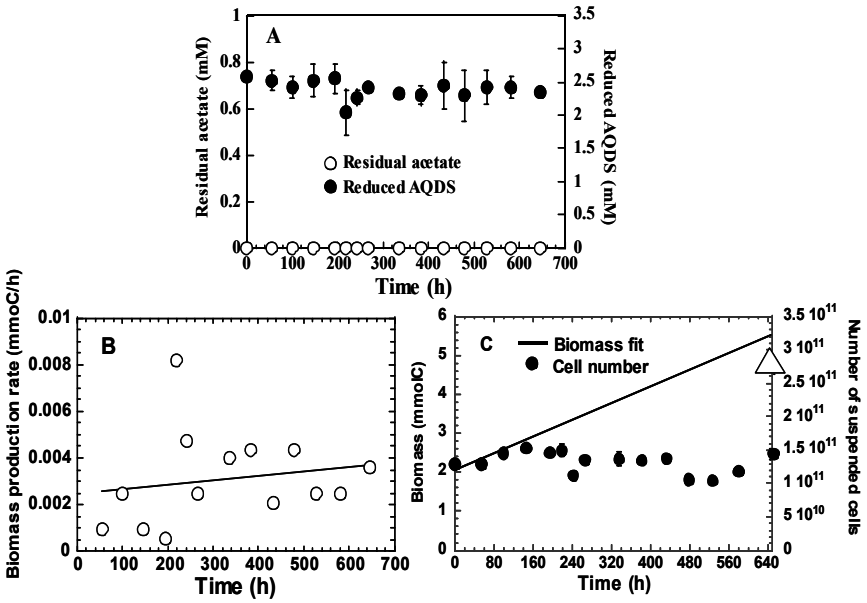

***Figure 2**. Substrate turnover and biomass data for G. metallireducens grown in continuous culture with 100 % internal biomass retention under acetate-limitation. **A**: concentration of reduced AQDS (●) and residual acetate (○). Bars indicate standard deviation (n=2); **B**: biomass production rate (r_x_t) (expressed in terms of mmol C_1-acetate). The line indicates the result of fitting equation 4 to the calculated production rates; **C**: comparison of measured cell numbers in the culture (●) to the fitted biomass (—), based on equation 1 and fitted growth parameters (Table 1), over time. (Δ) indicates the biomass after scrapping cells from surfaces in the fermentor.*

Ability to use alternative electron acceptors simultaneously

At the end of the experiments cells were growing at a rate of 0.001 h^{-1} in the AQDS-limiting retentostat and 0.00078 h^{-1} for the acetate-limiting retentostat (calculated from equation 2 divided by equation 1, and the fitted physiological parameters). The simultaneous, *in situ* expression of biochemical pathways for the utilization of alternative electron acceptors under these growth-conditions was tested in the presence of 150 µg/ml of the protein synthesis inhibitor chloramphenicol. 'Experienced' (in terms of ability to utilize AQDS) cells from the AQDS-limiting retentostat (at a final concentration 4 x 10^8 ml^{-1} cells) were able to reduce chelated Fe (III) in addition to AQDS. AQDS was not reduced over a period of one month with chloramphenicol added when inoculated at a much lower number (4 x 10^5 ml^{-1}) of 'experienced' cells (i.e. cells previously grown in the presence of AQDS), showing that the cells did not grow in the presence of the chloramphenicol, i.e. that the compound remained effective. In the corresponding experiments in the absence of chloramphenicol both growth and AQDS reduction was observed. Alternative electron acceptors (nitrate, Fe (III) citrate or ferrihydrite) were also completely reduced when chloroamphenicol was absent. The cell suspension (2 x 10^8 ml^{-1}) prepared from the acetate-

limiting retentostat (hence 'experienced' in terms of the presence of AQDS) reduced all tested alternative electron acceptors (chelated Fe (III) (Fe citrate)), amorphous Fe (III) (six line ferrihydrite) and nitrate; all in the presence of chloramphenicol) (Table 2). The amount reduced was at least 34 % of the amount reduced in the absence of chloramphenicol. This suggests that growth in the presence of AQDS does not repress and may even induce the capacity to reduce these other electron acceptors.

Table 1. Fitted maintenance energy demand (m_s) and maximum growth yield (Y_{xsm}) of *G. metallireducens* growing in retentostats under different limitations. Numbers in parenthesis indicate standard deviation.

Limitation	m_s (Cmol-acetate Cmol-biomass^{-1} h^{-1})	Y_{xsm} (Cmol-biomass Cmol-acetate^{-1})
AQDS	-0.0012 (±0.0023)	0.088 (±0.013)
Acetate	-0.016 (± 0.026)	0.053 (± 0.026)

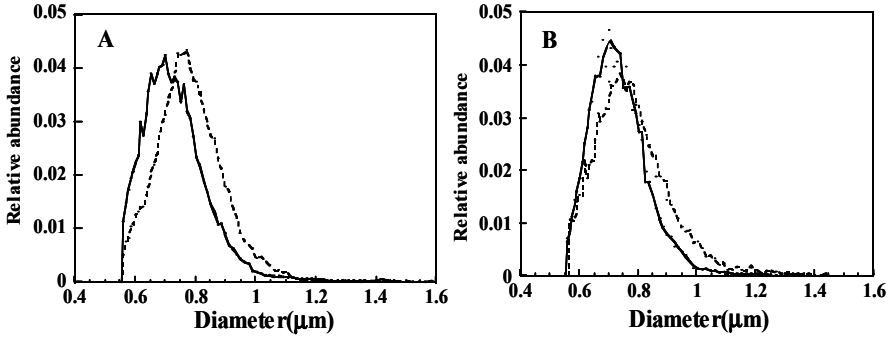

Figure 3. *Changes in the size distribution (diameter, in μm) of cells over time, expressed by relatively abundant cells in each different size out of total cell number.* ***A****: AQDS-limiting retentostat, distribution of cells over sizes at two time points,* i.e. *0 hour (–) and after 35 days (…);* ***B:*** *acetate-limiting retentostat, distribution of cells over sizes at two time points,* i.e.*, 0 hour (–) and after 34 days (…).*

Capacity for iron reduction

The capacity for the reduction of several forms of Fe (III) was determined for washed 'experienced' cells harvested from the AQDS-limiting retentostat at the end of the experiment. Three types of Fe (III) were used; chelated Fe (III) (iron citrate), amorphous Fe (III) oxide (ferrihydrite) and crystalline Fe (III) oxide (hematite). All forms were reduced by the microorganism, but the highest rates were observed with chelated Fe (III) (6.8 ± 1.3

x 10^{-9} μmol^{-1} cell^{-1} h^{-1}). Initial iron reduction rates with ferrihydrite and nanohematite were only 2.6 % and 0.062 %, respectively, of the rate with iron citrate (Table 3).

Table 2. Ability of 'experienced' cells harvested from AQDS-limited and of cells harvested from acetate-limited retentostats to use alternative electron acceptors, without inducing gene expression.

Treatment	AQDS-limited cells	Acetate-limited cells
AQDS	+	+
F6L	ND	+
Fe (III)citrate	+	+
Nitrate	ND	+

"+": at least 34 % of the electron acceptor was reduced after one week of incubation. ND: not determined; F6L, six-line ferrihydrite. Protein synthesis was inhibited by chloramphenicol.

DISCUSSION

Aspects of the physiology of iron-reducing microorganisms (mainly *Shewanella* and, less frequently, *Geobacter* species) have mostly been investigated using batch cultures (20, 35) and flow columns (32, 35). Recently, Lovley and coworkers began to apply chemostat cultivation to study aspects of the physiology of *Geobacter sulfurreducens* at growth rates of 0.03 h^{-1} and higher (6, 11, 12, 17). In the present study, the physiology of *G. metallireducens* was characterized under either growth-limitation by electron acceptor (AQDS) or by electron donor (acetate) in retentostats at growth rates down to 0.001 h^{-1}, i.e. much below 0.03 h^{-1}. Such low growth rates correspond to those observed in the field (31, 52).

Normal maximum growth yield and very low maintenance metabolism

Growth yield of the iron-reducers *Shewanella* and *Geobacter* as determined in batch culture are in the range of 1.3-6.1 x 10^{12} cells mol electrons^{-1} and 1.3-4.0 x 10^{12} cells mol electrons^{-1} respectively (20, 26, 27, 35). However, in these batch cultures growth rates are much higher than in the field, and it remained uncertain whether also under the latter conditions, growth yields of these organisms can be substantial. We here observed that they are: maximum growth yields obtained in this study were 0.4-1.8 x 10^{12} cells mol electrons^{-1}, i.e. only a little lower than those in batch culture. The maximum yield for *G. sulfurreducens* determined in chemostat with Fe (III) citrate as electron acceptor was 0.036-0.065 C-mol biomass/C-mol acetate (12), which is again comparable to the yields obtained in this study. Although iron and humic acid reduction are thermodynamically more favorable processes than sulfate reduction, the yields for *G. metallireducens* and *G. sulfurreducens* are comparable to those of sulfate reducers. Yield was 0.036 C-mol biomass/C-mol growth

substrate for *Desulfovibrio desulfuricans* while for *Desulfovibrio vulgaris* grown under lactate-limiting conditions, the yield was 0.10 C-mol biomass/C-mol lactate(33, 44). Our experiments show therefore that the maximum growth yields, i.e. the growth yield corrected for maintenance metabolism, are independent of the growth conditions. They do pertain to the ones that are more like those in the field and that lead to low growth rates.

Table 3. Initial iron reduction rate ($\mu mol^{-1}cell^{-1}h^{-1}$) of *G. metallireducens* on different forms of iron oxides under Fe (III) saturating conditions *.

	G. metallireducens	*S. putrefaciens*	Solubilities of iron oxyhydroxide (log $*K_{so}$)
Fe (III)citrate	$6.8\pm1.3x10^{-9}$ (100 %)	$4.1\pm0.2\ x10^{-9}$ (100 %)	
Ferrihydrite	$1.8\pm0.1x10^{-10}$ (2.6 %)	$6.5\pm\ 0.3x10^{-11}$ (1.6%)	0.52
Nanohematite	$4.2\pm0.7x10^{-12}$ (0.06 %)	$2.4\pm0.2x10^{-11}$ (0.6 %)	1.90

** Cells were harvested from an AQDS-limited retentostat and inoculated at approximately the same concentration as in the fermentor, i.e. 2.2 x 10^8 ml^{-1}. Average rate with standard deviation derive from triplicate determinations. Percentages in parentheses indicate the initial iron reduction rate for a particular Fe (III) source relative to the rate observed with Fe (III) citrate. The values for Shewanella putrefaciens were taken from the literature (2), where the cell density was approximately 3 x 10^8 ml^{-1}. Data on iron oxyhydroxide solubility are those of Bonneville et al. (2).*

Almost zero maintenance catabolism was observed for *G. metallireducens* in this study, independent of growth limitation. Growth-rate independent maintenance catabolism is the catabolism that persists at zero growth rate in order to keep the cell alive (47). This is determined by futile ATP consuming processes, ion leaks through the plasma membrane, as well as maintenance functionalities such as chaperoning and replacement synthesis of denatured proteins. For this reason it is also called maintenance energy demand (51). Recently, a maintenance energy demand of 0.058 C-mol acetate/C-mol biomass h^{-1} for *G. sulfurreducens* grown at 0.04-0.08 h^{-1} in chemostat cultures was reported (12), growth-rates substantially higher than the ones we report here for two independent experiments with different limiting substrates for *G. metallireducens*. Some care has to be taken in comparing the results from the study of Esteve-Nunez *et al*. (12) to ours, as Esteve-Nűňez *et al* grew *G. sulfurreducens* with fumarate or Fe (III) citrate as electron acceptor in the chemostat, whereas we used *G. metallireducens* with AQDS as electron acceptor in a retentostat. Attempts to grow *G. metallireducens* in chemostats were not successful due to wall-growth (data not shown).

A near zero maintenance demand at extremely low growth rates has not been observed in other studies, i.e. (1, 41, 49). The lowest reported maintenance energy demand for heterotrophic bacteria we encountered in literature was 0.005 C-mol mannitol C-mol $biomass^{-1}$ h-1 for aerobically grown *Paracoccus denitrificans* (48), where mannitol is an aerobic substrate much richer in free energy than the acetate in our anaerobic studies (51).

The maintenance requirements seem to be very low when microbial specific growth rate is lower than 0.01 h^{-1} (10). In our studies under environmentally relevant conditions leading to growth rates as low as 0.001 h^{-1}, there was no maintenance energy demand for *G. metallireducens*. Decreases in maintenance metabolism with decreasing growth rate for *Geobacter* species could allow them to cut down on their maintenance processes when times get tough. It may also be that these organisms simply have a much lower maintenance metabolism than most other organisms.

The ecological advantage of having a low maintenance energy demand and of being flexible with respect to electron acceptors

Three different explanations have previously been proposed for the dominance of Geobacters in iron-reducing subsurface environments (29): *G. metallireducens* is capable of (i) using acetate as growth substrate, a major intermediate in subsurface (23), (ii) chemotaxis towards high concentrations of Fe (II), which should be indicative of sources of Fe (III) oxides (5), as well as (iii) nitrogen fixation (16). However, Geobacters are not only found as predominant bacteria in oligotrophic subsurface environments. They have also been found in a landfill leachate-polluted aquifer (36). In this aquifer, ammonium concentrations were as high as 20 mM (45). Therefore, the ability for nitrogen fixation is not the sole reason for the high abundance of Geobacters in iron-reducing aquifers.

The observed low maintenance energy requirement provides an alternative, or additional, explanation for the dominance by *Geobacteraceae* in iron-reducing environments, as it allows growth at low concentrations of the growth-limiting compound. We arrive at this explanation by continuing the common analysis in which growth-rate independent energy maintenance demand constitutes a constant component of the rate of catabolism. The specific growth rate, μ (in h^{-1}), is then described by Westerhoff and van Dam (51):

$$\mu = \left(J_S - m_S\right) \cdot Y_{xsm} \qquad [5]$$

J_S equals the substrate uptake flux (mmol substrate/(biomass unit*h). Its dependence on the extracellular substrate concentration can be described by the Michaelis-Menten or Monod equation:

$$J_S = V_{\max} \cdot \frac{[S]}{K_S + [S]} \qquad [6]$$

in which: V_{max} = maximum rate, K_S = affinity constant (mM). By setting $\mu = 0$, inserting equation [6] in [5] and re-arranging, an expression for the substrate concentration above which growth is observed, is obtained:

$$[S]_{\min imum\ growth} = \frac{m_S \cdot K_S}{V_{\max} - m_S} \qquad [7]$$

This expression shows that a lower maintenance energy requirement allows for growth at lower substrate concentrations.

Yet another potential explanation of why Geobacters are generally encountered in iron-reducing subsurface environments may relate to their flexibility in the use of electron-acceptors. We here showed that *G. metallireducens* grown on the humic acid analog AQDS at low growth rates is able to utilize alternative electron-acceptors simultaneously, even if the humic acid analog is not growth-limiting. This may help Geobacters to deal with varying redox conditions and rapidly scavenge electron-acceptors that allow for a higher growth yield per electron transferred under slow-growth conditions. Adaptation of slow-growing *Geobacter* through altered gene expression is not even always necessary, as we showed in the present study (Table 2). In contrast, cells of *G. metallireducens* grown on Fe (III) at maximal growth rate in batch cultures did not have the ability to reduce nitrate although cells grown on nitrate were able to reduce iron (13), suggesting that fast-growing cells using Fe (III) as electron acceptor have less flexibility towards changing redox conditions.

Capacity for iron reduction

Species from the iron-reducing genus *Shewanella* are hardly found as dominant iron reducers in subsurface environments. Yet the kinetics of iron reduction has been mainly studied for *Shewanella* species, because these facultative anaerobic microorganisms are much easier to cultivate in the laboratory than strict anaerobes such as *G. metallireducens*. In such studies, *Shewanella* has been grown aerobically in batch culture until the early stationary phase after which cells were collected and experiments on their iron-reducing capacities were performed (2, 22, 34). These growth-conditions do not represent the conditions in which iron reduction takes place naturally. Retentostat cultivation can mimic natural conditions and high quantities of cells are easily obtained. We ere showed that the device of retentostat is suitable for studying the physiology of iron reduction by strict anaerobes like *Geobacter*.

Solubility and surface properties of Fe (III) oxyhydroxides are additional factors influencing mineral surface-associated cell activity (32), as they determine the bioavailability of Fe (III) (21, 35, 53). The maximum specific rate of reduction correlated positively with the solubility of Fe (III) oxyhydroxides for *S. putrefaciens* (2), and for *G. metallireducens* (this study). Overall rates of reduction of Fe (III) citrate and ferrihydrite as well as the rates of reduction for ferrihydrite relative to Fe (III) citrate were similar (3 %, and 2 % for *S. putrefaciens* and *G. metallireducens* respectively), however that of hematite was a factor 10 lower for *G. metallireducens*.

CONCLUSIONS

The application of the retentostat has allowed the study of Geobacters at very low growth rates that are representative for their natural environment. At these low growth rates we observed that maintenance catabolism was virtually absent, suggesting that these organisms are capable of reducing the dissipation of free energy they require for maintaining the living state. In addition we observed a high versatility with respect to changing environmental conditions in terms of different electron acceptors. These results may help understand why Geobacters dominate many natural iron-reducing environments. Retentostat experiments as described here could easily be combined with transcriptomic, proteomic and metabolomic analyses in order to further enhance insight in microbial functioning at the low growth rates encountered in natural habitats.

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Chapter 7

General discussion

In this chapter, the results of research presented in the previous chapters are discussed as follows: (i) relation between Geobacters (iron reduction) and biodegradation, and how to use this information in monitoring and directing bioremediation, (ii) relations between the composition of iron-reducing communities and environmental conditions, (iii) importance of iron reduction and of the microorganisms directly performing this process, for ecosystem functioning.

The role of Geobacters in the biodegradation of aromatic compounds under iron-reducing condition Biological treatments (bioremediation), particularly the ones using microorganisms, are well-recognized measures to handle pollutants. Examples include composting and the use of bioreactors. 'Bioremediation' has been defined as 'the elimination, attenuation or transformation of polluting or contaminating substances by the use of biological processes to return the environment altered by contaminants to its original condition' (41). For groundwater pollution in the subsurface, especially when aromatic compounds are the main contaminants, *ex situ* treatment, requiring the pumping up of groundwater is common practice. However, this is expensive. The natural attenuation of pollutants in the subsurface due to *in situ* microbial activities is drawing much attention as it is less expensive than active treatment. Particularly the rapid advances with respect to the attenuation of aromatic hydrocarbons coupled to microbe-mediated iron reduction (1, 10, 39) hold promise for the future regarding the control (monitoring, enhancement) of pollution in the subsurface.

Microbial iron reduction using Fe (III) as terminal electron acceptor is an ubiquitous and important redox process in anoxic environmental settings, i.e. in marine (12, 18, 25) and freshwater sediments (1, 16, 26, 55, 56). Microbial iron reduction is actively coupled to the oxidation of aromatic compounds including toxic toluene and benzene (8, 10, 35, 37, 39, 54, 59). *Geobacter* species, which are all able to reduce iron (34), are frequently encountered in aromatics-contaminated iron-reducing environments (20, 31, 48, 52, Chapter 2, 3). *Geobacter metallireducens* and *G. grbiciae* are capable of mineralizing toluene to carbon dioxide (12, 40). Together with a recently described isolate of *Rhodoferax ferrireducens* (15), these two species are so far the only species capable of degrading toxic aromatic compounds as a pure culture under iron-reducing conditions.

Members of the *Proteobacteria* dominated in clone libraries retrieved from the Banisveld landfill-leachate polluted aquifer. Particularly, the iron-reducing family *Geobacteraceae* formed a considerable part of the microbial community in this aquifer (Chapter 2). Additional research revealed that the presence of some *Geobacter* phylotypes

clearly correlated with attenuation of dissolved organic carbon (DOC), benzene (B), toluene (T), ethylbenzene (E), xylene (X) and naphthalene (N) (Chapter 3). This correlation might indicate that these *Geobacter* phylotypes are involved in the process of aromatic compounds degradation in the Banisveld landfill leachate-polluted aquifer. They could be directly degrading aromatic compounds or intermediates formed in the degradation. Unfortunately, we did not isolate representatives of these phylotypes in pure culture (chapter 5), and thus could not test their ability to degrade aromatic compounds.

The 16S rRNA gene-based detection of Geobacters alone does not provide sufficient evidence to conclude whether BTEX degradation can indeed occur. Not all Geobacters species are capable of degrading aromatic compounds. The detected Geobacters based on 16S rRNA genes may also include Geobacters present in the particular environment but unable to degrade aromatic pollutants. Concluding on the presence of Geobacters also requires caution, as the primers employed in this study, do not only amplify *Geobacter* 16S rRNA genes (chapter 3). Therefore, more specific primers targeting Geobacters should be developed, possibly targeting *Geobacter*-specific functional genes, such as genes relating to nitrogen fixation (21) or to iron reduction (Röling, unpublished data). With respect to BTEX oxidation, the detection of functional genes involved in aromatic compound degradation should be taken into account even though the research on the genetic aspects of anaerobic aromatic compound degradation is still in its infancy. For instance, the genes for the three subunits of benzylsuccinate synthase (bssCAB), the enzyme initiating anaerobic toluene degradation by converting toluene to (R)-benzylsuccinate, have been characterized (27, 50). Thus, primers targeting benzylsuccinate synthase could in principle provide more direct information on the potential for toluene degradation. *G. metallireducens* contains these genes (27, 50). Current information suggests that the bssA genes, as well as several other genes relating to anaerobic toluene degradation, are well-conserved among anaerobic nitrate, iron and sulfate-reducing toluene degraders (Röling, unpublished data; Lueders, personal communication). In addition to DNA-based techniques, mRNA based analyses can be taken into account for microbial activity determination, i.e. RT-PCR, and microarray-based genomic techniques (60). mRNA based studies will be more revealing vis-à-vis understanding the actual involvement of particular microorganisms in the process of aromatic biodegradation.

Despite the widespread application of culture-independent analysis in microbial ecology, studies on pure cultures, or defined consortia, are still crucial for understanding the ecophysiology of iron-reducers as well as for further development and interpretation of the molecular approaches. For instance, physiological characterization of *Geobacter metallireducens* by retentostat cultivation (chapter 6) provided insight into why Geobacters might often be dominating the subsurface (i.e. due to their extremely low maintenance energy, and flexible use of alternative electron acceptors). In addition to *Geobacter spp*., enrichments and isolations revealed iron-reducing *Serratia, Desulfitobacterium, Clostridium* and *Rhodoferax spp*. in the plume of pollution (Chapter 4). *Clostridium* and

Rhodoferax spp. are potentially capable of the degradation of aromatic compounds (13, 15, 46). Thus, Geobacters are possibly not the (only) microorganisms degrading aromatic hydrocarbon in the aquifer downstream of Banisveld landfill. In fact, a non-*Geobacter*, toluene degrading iron-reducer has been isolated from this research location (Weelink, personal communication). The observed diversity of iron reducers, both within the family *Geobacteraceae* (chapter 3) and within the domain *Bacteria* (chapter 4) also indicates the existence of a pool of functionally redundant (with respect to iron-reduction) microorganisms, and consequently such a pool may allow a quick response (i.e. growth of particular types of iron-reducers) to changing environmental conditions, i.e. following depletion of certain types of Fe (III) oxides or electron donors.

Intrinsic aromatic hydrocarbon degradation occurs in iron-reducing subsurface environments, where Geobacters play an important role in this process (11,49, 24). Usually the process is slow but can be stimulated and/or enhanced for soil and groundwater remediation by increasing the solubility of Fe oxides. As Fe (III) oxides are the most abundant form of the element iron in natural environments, their bioavailability to iron-reducing microorganisms may set the rate of iron reduction. Indeed, the bioavailability of Fe (III) oxides has been reported as the limiting factor in the landfill leachate-polluted aquifer Vejen, in Denmark (2).

For the well-studied iron-reducer *Shewanella putrificans*, the maximum specific rate of reduction (V_{max}) correlated positively with the solubility of four different Fe (III) oxides (ferrihydrite, lepidocrocite, goethite, hematite), with the highest values for ferrihydrite and amorphous Fe (III) oxide and the lowest for hematite (6). The initial iron reduction of *G. metallireducens* (chapter 6) is comparable to that of *S. putrefaciens* (6), therefore the rate of microbial iron reduction might be more dependent on the solubility of Fe (III) oxides than on microbial species. This may indicate that kinetic data obtained from studies on *Shewanella* can be applied to environments in which Geobacters dominate. This is of advantage in obtaining kinetic data for mathematical modeling of bioremediation processes, for example to determine the sustainability of this process or to predict what will happen upon human intervention: *Shewanella* is much easier to work with than *Geobacter*, since it tolerates oxygen very well.

The addition of chelators or electron shuttles is known to increase the utilization of Fe (III), which is coupled to the oxidation of organic matter (5). Modeling has indicated that the contribution of chelation to total iron reduction rate can be considerable under natural conditions, in contrast to electron-shuttling via humic acids. (Röling, unpublished). These results can be applied in designing monitoring and bioremediation strategies (i.e. monitoring of chelator concentrations).

As described in chapter 6, the strain corresponding to the dominant *Geobacter* phylotype, indicative of pollution and occurring in the part of the aquifer with the highest rates of organic matter biodegradation, was not recovered in batch culture, despite the provision of various growth-conditions. Studies on this strain would have provided valuable

data with respect to its potential and capacity in natural attenuation. Possibly, this *Geobacter* might still be enriched by using a Banisveld subsurface-simulating retentostat, which can create '*in situ* living conditions', at a low substrate concentration and low growth rate. It may well be that this *Geobacter* is only capable of slow growth and that the growth conditions in batch culture are unfavorable due to competition by faster-growing bacteria for substrates ulitization competition. The addition of extracts from sediments, or growth on filter-sterilized groundwater, may aid in its growth during the enrichment by providing essential growth factors, such as vitamins. Further physiological characterization of the dominant *Geobacter* as well as of retrieved isolates (chapter 4; *Geobacter, Serratia, Rhodoferax*), both in terms of their kinetics of utilization of organic matter such as BTEXN (benzene, toluene, ethylbenzene, xylene and naphthalene) pollutants in the presence of various types of iron oxides and in terms of the mechanisms by which they access insoluble iron oxides, will provide further insight in the potential roles of these organnisms in the natural attenuation of aromatic hydrocarbons.

Composition of iron-reducing communities in relation to environmental settings

There is debate in the scientific community about which genera of iron-reducing microorganisms is environmentally most important (*Geobacter* and/or *Shewanella*). The results from this study and other studies (45, 47) indicate that the composition of the iron-reducing community is environmentally dependent, although quite often Geobacters are observed to dominate circumneutral pH subsurface settings in terms of cell number.

Members of *Geobacteraceae (δ-Proteobacteria)* have been observed frequently in iron-reducing subsurfaces contaminated by petroleum (3), landfill leachate (31, 48, Chapter 2, 3) or metal (4, 14, 19, 43)at neutral pH. The ability of Geobacters to colonize and dominate such environments has been suggested to relate to (i) chemotaxis towards Fe (II) and Mn (II) in natural environments, in which insoluble Fe and Mn oxides are dominant forms (9, 30), (ii) the capability of using the key intermediate acetate in the subsurface (36), (iii) the extremely low free-energy demand for maintaining their living state, which allows them to grow at lower substrate concentrations than other species (Chapter 6 of this thesis), (iv) the flexibility in the immediate use of alternative electron acceptors for cells grown on AQDS as electron acceptor without the requirement to synthesize new enzymes (Chapter 6), (v) tolerance of relatively high oxygen concentrations (10%), which usually are encountered at the interface of anoxic and aerobic zones (32), and (vi) possession of genes involved in nitrogen fixation, which may assist them to compete effectively in nitrogen-poor subsurface environments (21). (Combinations of) these properties may result in Geobacters having ecological advantage(s) over other iron reducers (i.e. *Shewanella*) in many subsurface settings, allowing them to become dominant. On the other hand, to a particular environment, not all the properties mentioned above may apply. For example the ability for nitrogen-fixation is unlikely to contribute to the dominance of Geobacters in the ammonia-rich aquifer contaminated by the Banisveld landfill leachate.

Members of the genus *Shewanella (γ- Proteobacteria)* are the most intensively studied iron-reducing microorganisms. They are facultatively anaerobic bacteria and are found in a variety of sedimentary environments (57). *Shewanella* have been used most for kinetic characterization, as they are easy to handle (17, 33, 42). However, species from *Shewanella* have not been shown to contribute substantially to iron reduction in the vast majority of iron-reducing environments or to be able to degrade aromatic contaminants (38). To date, there are no indications for the importance of *Shewanella* in polluted, anaerobic subsurface settings. *Shewanella* was not detected in the Banisveld landfill polluted aquifer (chapter 3) nor in metal-contaminated subsurface environments (51) and are only present in low numbers in the Scheldt estuary (Chapter 5). There might be several reasons causing the absence or low abundance: (i) most *Shewanella* cannot use acetate, the key intermediate in the anaerobic degradation of organic matter(36); (ii) when direct contact is impossible, *Shewanella* can indirectly utilise Fe (III) via secreted extracellular compounds (shuttles, chelators). This mode of living (i.e. the production of shuttles and chelators coupled to losses of these compounds to the aqueous environment) might be more suitable to free-energy consuming than a living style in which chemotaxis is used to detect fresh sources of Fe (III) oxides (38).

While Geobacters usually dominated in circumneutral pH environments, *Anaeromyxobacter spp*. were recently found to be dominating in acidic subsurface environments contaminated with uranium (45, 47). Other iron-reducing microbes have been recovered from acidic environments, i.e., an iron-reducing acidophilic thermophile – strain SJH (23), *Acidiphilium cryptum* JF-5 (28), *Acidiphilium spp.* (22), *Thiobacillus thiooxidans* and *Sulfolobus acidocaldarius* (7). Thus, environmental pH seems to be one of factors affecting the composition of iron reducing communities. This is not surprising as the solubility of iron and its ligands are functions of pH.

However also at neutral pH, Geobacters are not always the dominating iron-reducers, as shown in chapter 5. The environmental conditions in the Scheldt estuary are much more complex and variable than in most subsurfaces, and certainly in the subsurface we studied here, as it is heavily influenced by continuous input of freshwater carrying a complex mixture of compounds (i.e. organic pollutants, ammonium, metals). In the Scheldt estuarine sediments, the presence/availability of a wide range of electron donors and of various forms of Fe (III), as well as of other electron acceptors, possibly allows a phylogenetic diverse and physiologically flexible range of iron reducers (*Ralstonia, Clostridium, Geobacter, Shewanella, Geothrix, Anaeromyxobacter* and *Alkaphilius)* to colonize the sediments. These iron reducers have different mechanisms to access Fe (III) oxides. These mechanisms involve (i) direct contact (all iron reducers), (ii) indirect reduction via electron shuttling compounds (i.e., *Geothrix fermentans* (44), *S. putrefaciens* (29) and *S. algae* (53)) or (iii) chelation of Fe (III) and subsequent reduction. Regarding the third stratgies, organic matter such as citrate and oxalate from the input of wastewater and microbial activity can function as ligands (chelators) to increase the effective solubility

of Fe (III). By facilitating transport to where iron reducers occur, this provides rapid access of Fe (III) oxides to those reducers (6).

Our study has increased the knowledge with respect to the types of iron reducers present in the Scheldt estuary and the understanding of their metabolic behaviour with respect to relevant environmental settings. However, further investigation of microbial iron reduction in such estuaries is required as the geochemistry in estuaries is more complicated than that in subsurfaces such as the Banisveld landfill aquifer. In particular we recommend the following: (i) Characterisation of the organic matter present in the Scheldt estuary should be carried out, as its composition might be an important factor controlling the microbial iron reduction, as well as the composition of the iron-reducing microbial communities. (ii) It should therefore it be interesting to know whether the kinetics of the *Ralstonia* and *Clostridium* with respect Fe (III) oxides are comparable to the kinetics of Fe (III) reduction by *Shewanella* and *Geobacter*. *Shewanella* has an iron reduction rate that is comparable to that of *Geobacter* (6, chapter6), however, these two groups of bacteria are not dominant in Scheldt estuary sediment. Instead, *Ralstonia* and *Clostridium* make major contributions to the iron-reducing communities found in the sites studied. These species appear to use Fe (III) solely as an electron sink enabling ATP formation via substrate-level phosphorylation, in contrast to *Shewanella* and *Geobacter* which gain energy to support growth from the Fe (III) reduction process itself. (iii) Detection of specific iron reducers in the Scheldt estuary is only indicative for their presence, i.e. it does not constitute proof that they also contribute significantly to the actual reduction of Fe (III). Indeed, considerable flexibility was observed with respect to the use of electron acceptors. Therefore, while the detected and isolated strains are capable of iron reduction, they may not contribute significantly to iron-reduction under *in situ* conditions. It is important that more strains are isolated and their actual Fe (III) reduction activities are assessed.

Intergating of iron-reduction into ecosystem functioning

We observed that members of *Geobacteraceae* were dominant components of iron-reducing microbial communities correlating to aromatic compound degradation in the Banisveld landfill leachate-polluted aquifer. By contrast, in estuary sediments, in addition to the well-known iron reducers (*Geobacter, Shewanella, Geothrix, Anaeromyxobacter* and *Alkalphilius*), *Ralstonia* and *Clostridium* made major contributions to the iron-reducing communities. Kinetic or flux studies are required in order to determine to which degree the various groups are contributing to environmental iron reduction rates.

To understand ecological processes (or ecosystem functioning) better, in the future the system as a whole (community structure, activities of individual members and interactions between the different members, as well as with their environment (the chemical and physical structure of the environment) should be studied, i.e. not just its parts, or just those parts which are now considered important (i.e. the pollutant degrading microorganisms in biodegradation, the iron-reducing microorganisms in iron-reduction).

An ecological variant of metabolic control analysis revealed that control on a certain flux can be distributed over several groups and control over intermediate concentrations is always shared (58). Assuming a simple network of fermenting microorganisms interacting with iron-reducers via the intermediates hydrogen and acetate, we deduced that the flux of organic matter and iron-reduction mainly resided with the fermenting bacteria, not with the iron-reducing bacteria (Röling et al., unpublished data). Therefore, not only the activity of iron-reducing (micro) organisms should be considered when studying iron-reduction in an ecological context, but also the activity of microorganisms that affect the activities of these microorganisms by producing or interfering with their growth substrates or their survival (i.e. predators, competitors).

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Summary

Microbial iron reduction plays a major role in biogeochemical cycling of iron and other metal elements, as well as in the oxidization of compounds originating in human activity on this planet. This includes primary production and anthropogenic organic matter in the anaerobic subsurface. Moreover, dissimilatory Fe (III) iron reduction was probably one of the first important respiratory pathways and may have greatly influenced early biological and biogeochemical evolution on Earth. The geochemical and ecological importance of microbe-mediated reduction of iron oxyhydroxides in subsurface systems is widely recognized, but little understood.

The objective of the research described in this thesis is to increase insight in the mechanisms of microbial iron reduction in the environment. We study the composition of iron-reducing communities, and their functioning in two contrasting environments, i.e. an aquifer polluted by a neighbouring landfill, and the estuarine sediments. To achieve this goal, both field-scale hydrochemical measurements and laboratory experiments were performed. Comprehensive knowledge on the presence and functioning of iron-reducing microbial communities was obtained through culture-independent 16S rRNA-based analyses, and through physiological characterization of iron-reducing microorganisms recovered from nature. The physiological characterization extended to an anaerobic model strain, *Geobacter metallireducens*, grown in batch or retentostat.

In **Chapter 1** general introductions are given to eight aspects of microbial iron reduction, i.e. (i) abiotic and biotic iron reduction, (ii) the global importance of enzymatic iron reduction, (iii) where and how iron reduction occurs, (iv) isolated microorganisms capable of reducing iron oxides, (v) how iron reducers access iron oxides, (vi) the relationship between bioavailability of Fe (III) oxides and iron reduction, (vii) the electron donors usually encountered in the subsurface and (viii) cultivation-independent assessment of microbial communities in iron-reducing environments.

Iron reduction is often a major redox process in groundwater polluted by a neighbouring landfill. Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer (Banisveld, The Netherlands) are described and discussed in **Chapter 2**. In clean reference locations two redox processes were observed. Denitrification was the dominant redox process above the plume, while beneath the plume iron reduction occurred. Degradation of organic contaminants occurred under iron-reducing conditions in the plume of pollution. Numerical analysis of 16S rRNA gene-based DGGE (denaturing gradient gel electrophoresis) profiles (*Bacteria* and *Archaea*) and sequencing of cloned 16S rRNA genes revealed a considerable difference between the microbial community structure inside and outside the plume of pollution. Sequencing of cloned 16S rDNA supported the DGGE data. Relations between dominant redox processes and molecular identities were observed. Members of the *β-Proteobacteria* dominated in clone libraries. However, *β-Proteobacteria* (*Acidovorax*, *Rhodoferax*) in the

iron-reducing plume differed considerably from those found upstream (*Gallionella*, *Azoarcus*) in clean, iron-reducing groundwater. In the iron-reducing plume of pollution, members of the iron-reducing family *Geobacteraceae* strongly contributed to microbial communities, accounting for approximately 25% of the total *Bacteria* population count (MPN_PCR).

Geobacteraceae have also been found as dominant members of microbial communities in other carbon-rich iron-reducing subsurface environments. So far, only members of the Geobacters and *Rhodoferax* have been implicated in the degradation of monoaromatics under iron-reducing conditions. Therefore, the findings described in Chapter 2 led us to investigate the relations between community composition of the iron-reducing *Geobacteraceae,* pollution levels and occurrence of pollutant degradation in more detail (**Chapter 3**). Using cultivation-independent 16S rRNA gene based DGGE profiling and sequencing, we observed a considerable difference in *Geobacter* community composition between the plume and the unpolluted parts of the aquifer, suggesting pollution has selected for specific *Geobacter* species. The DGGE profiles of polluted groundwater taken near the landfill (6-39 m distance) clustered together, whereas the profiles from less-polluted groundwater taken further downstream did not fall in the same cluster. Furthermore, the high *Geobacteraceae* diversity points to the existence of a large pool of iron reducing Geobacters in the Banisveld landfill aquifer. More *Geobacter spp* with different physiological abilities might be present, suggesting that a larger number of organic compounds might be degraded. Several bands in the DGGE profiles were indicative of either the redox process or the level of pollution. The high intensity of some pollution-indicative bands corresponded to the part of the aquifer with a relatively high rate of attenuation of organic micropollutants and reactive dissolved organic matter.

Molecular fingerprinting gives rapid insight in which microorganisms are present, but not necessarily in their physiological potential and capabilities. In order to enhance knowledge on natural attenuation of landfill leachate under iron-reducing conditions further, iron-reducing consortia and isolates from the Banisveld landfill-leachate polluted aquifer were recovered (**Chapter 4**). Iron reducers*,* including *Serratia, Clostridium,* and *Geobacter spp.* were isolated. *Rhodoferax sp.* was predominantly present in dilution-to-extinction enrichments. This indicates that a diverse range of iron reducers other than Geobacters alone, is present in this leachate-polluted aquifer. The number of culturable iron-reducing bacteria was low at 80-140 per gram sediment in polluted sediments, but more than five times higher than in non-polluted sediment. Although it is often claimed that *Geobacter* species, in contrast to many other microorganisms, are easily culturable, the species that revealed dominant bands in DGGE as well as a high contribution of its 16S rRNA genes to clone libraries, as reported in chapters 2 and 3, was not retrieved. This despite the use of a wide range of incubation conditions (with respect to pH, temperature and sources of iron (III) and carbon). Instead, other *Geobacter* species were enriched and isolated.

Physiological aspects of *Geobacteraceae* were studied by culturing *G. metallireducens*, in a habitat-resembling retentostat, which mimics *in situ* conditions with a very low growth rate ($0.008h^{-1}$) (**Chapter 6**). The maximum growth yield was 0.05 and 0.09 C-mol biomass per C-mol acetate. The iron-reducing capacity of *G. metallireducens* was comparable to that of *Shewanella putrefaciens*, the highest initial iron reduction rate being observed for citrate-chelated Fe (III) (i.e. $7x10^{-9}$ μmol $cell^{-1}$ h^{-1}), whilst the rates in the presence of amorphous ferrihydrite and crystalline nanohematite were 40 and 1500 times lower, respectively. The observed very low maintenance energy demand (the lowest ever reported for a heterotrophic bacterium) and the ability to use alternative electron acceptors readily, without the requirement for synthesis of new proteins, provides alternative or/and additional explanations for why Geobacters are ubiquitous and dominant microorganisms in many iron-reducing subsurface settings.

Compared to aquifers (Chapter 2, 3, and 4), shallow estuarine sediments, such as found in the Scheldt, have unique, contrasting biogeochemical characteristics, i.e., high spatial (i.e. with depth on the cm scale) and temporal dynamics in redox processes. Like the Banisveld-landfill polluted aquifer, the Scheldt ecosystem has been influenced heavily by anthropogenic activities with complicated organic matter input. In order to address the potential for enzymatic iron reduction for two locations, freshwater Appels (Belgium) and brackish Waarde (The Netherlands), both in the Scheldt estuary, we combined field-scale geochemical measurements with laboratory experiments on the associated microbiology (**Chapter 5**). The community structure of the iron-reducing microbial population was complex at both sites. Iron reducing *Geobacter, Shewanella, Geothrix, Anaeromyxobacteria* and *Alkaliphilus spp.* were encountered but were not dominant (<1% of the cell counts) in either site. *Ralstonia and Clostridium spp.,* which were obtained as pure cultures, appear to be dominant iron reducers in the Scheldt estuary. A smaller contribution came from *Bacteroidetes* (22% for Appels, and 20% for Waarde) and *Actinobacteria* (5%, for Appels) or *Fusobacteria* (7% for Waarde). The presence of diverse and abundant (4.6×10^6, 2.4×10^5 cells g^{-1} sediment for Appels and Waarde, respectively) iron reducers, their versatility with respect to pH and temperature, their apparent ability to use alternative electron acceptors and donors, and substantial Fe (III) bioavailability strongly indicate a considerable microbial iron reduction potential. The dynamics and diversity in electron donors and in redox cycling of electron acceptors (i.e. formation of bioavailable Fe (III) phosphate) may have led to the presence of a physiologically diverse range of iron reducers and may have selected against strictly anaerobic *Geobacteraceae*.

In **Chapter 7** the results reported in the preceding chapters are discussed from three persepctives, i.e. (i) the role of Geobacters in the biodegradation of aromatic compounds under iron-reducing conditions, (ii) composition of iron-reducing communities in relation to environmental settings, (iii) iron-reduction as one of the aspects of ecosystem functioning.

Presence of some *Geobacter* phylotypes clearly correlated with attenuation of dissolved organic carbon (DOC) and BTEX in the Banisveld landfill-leachate polluted aquifer, suggesting that *Geobacter* phylotypes are involved in the degradation of aromatic compounds. In addition to dominant Geobacters species, iron-reducing *Serratia, Desulfitobacterium, Clostridium* and *Rhodoferax spp*., are also encountered in the plume of pollution. This phenomenon reflects a pool of functionally redundant (with respect to iron-reduction) microorganisms, which may or may not be involved in the anaerobic degradation of aromatics. Instead of methods addressing 16S rRNA genes as employed in this study, methods for detection and monitoring of functional genes responsible for aromatic compound degradation should now be implemented. Only then the basis of bioremediation by spiking the more active organisms should become an option. In anticipation, some such options for *in-situ* bioremediation of soil and groundwater are discussed, the addition of chelators or electron shuttles that should increase the utilization of Fe (III) oxides, a factor limiting iron reduction.

The composition of iron-reducing communities has been suggested to relate to environmental settings. Geobacters are ubiquitously encountered in iron-reducing subsurfaces, contaminated by petroleum, landfill leachate, or metal at neutral pH. The reasons why Geobacters are frequently observed in such environments are sought in their genetic and physiological properties. *Geobacter* and *Shewanella*, model strains for research pertaining to iron reduction, however are not found to be dominant in acidic environments and the pH neutral Scheldt estuary studied here. The presence/availability of both a wide range of electron donors, and a range of electron acceptors including Fe (III) in the Scheldt estuarine sediments, may enable a phylogenetic diverse and physiologically flexible range of iron reducers (*Ralstonia, Clostridium, Geobacter, Shewanella, Geothrix, Anaeromyxobacter* and *Alkaphilius)* to colonize the sediments.

Iron-reduction is one of the parts in ecosystem functioning as this process closely relates to the fate of organic matter, metal and nutrient cycling. In order to understand ecological processes better, the system as a whole (community structure, activities of individual members, interactions between the different members, interactions with their environment, as well as the chemical and physical structure of that environment) should all be considered for a more advanced understanding of iron reduction in those processes.

Samenvatting

Samenstelling en functioneren van ijzerreducerende gemeenschappen in twee contrasterende omgevingen, n.l. vuilstort percolaat-vervuilde aquifer en estuarium sedimenten

Microbiële ijzerreductie speelt een belangrijke rol in de biogeochemische ijzer cyclus, en in de oxidatie van organisch materiaal, zoals antropogene stoffen, onder zuurstofloze condities in de ondergrond. Waarschijnlijk was ijzerreductie het eerste ademhalingsproces op de vroege aarde. Het geochemische en ecologische belang van microbiële reductie van ijzer oxyhydroxydes in de ondergrond wordt onderkend, maar is weinig begrepen.

Het doel van het onderzoek, zoals beschreven in dit proefschrift, was het verkrijgen van meer inzicht in de mechanismen van ijzerreductie, door het bestuderen van de samenstelling van ijzerreducerende gemeenschappen, en hun functioneren, in twee contrasterende omgevingen, namelijk een aquifer vervuild door een naburige vuilstort en estuarium sedimenten. Om dit doel te bereiken zijn hydrochemische metingen gedaan in het veld, terwijl in het laboratorium de ijzerreducerende gemeenschappen zijn onderzocht. De microbiologie is onderzocht aan de hand van cultivatie-onafhankelijke 16S rRNA gen gerichte analyses en de fysiologische karakterisering van stammen geïsoleerd uit het veld. De fysiologische karakterisering omvatte ook een model ijzerreducerend organisme, *Geobacter metallireducens*, gegroeid in batch en retentostat.

Hoofdstuk 1 geeft een algemene inleiding en behandeld acht aspecten die belangrijk zijn met betrekking tot microbiële ijzerreductie, (i) abiotische en biotische ijzerreductie, (ii) het algemene belang van enzymatische ijzerreductie, (iii) waar en wanneer ijzerreductie optreedt, (iv) geïsoleerde ijzerreducerende microorganismen, (v) hoe ijzerreduceerders in staat zijn ijzer oxyhydroxydes om te zetten, (vi) de relatie tussen de bio-beschikbaarheid van ijzer oxyhydroxydes en ijzerreductie, (vii) de elektronendonoren die men aantreft in de ondergrond en (viii) cultivatie-onafhankelijke karakterisering van microbiële gemeenschappen in ijzerreducerende omgevingen.

IJzerreductie is vaak het dominante redox proces in grondwater vervuild door organisch materiaal. De relatie tussen microbiële gemeenschap structuur en hydrochemie in een aquifer vervuild door een naburige vuilstort (Banisveld, Boxtel) worden beschreven in **hoofdstuk 2**. In schone referentie locaties werden twee redox processen aangetroffen: denitrificatie boven de pluim van vervuiling, ijzerreductie eronder. In de pluim zelf werd organisch materiaal onder ijzerreducerende condities afgebroken. Numerieke analyse van denaturing gradiënt gel electroforese (DGGE) profielen van 16S rRNA gen fragmenten van *Bacteria* en *Archaea*, en sequensen van gekloneerde 16S rRNA genen, lieten een duidelijk verschil zien tussen de microbiële gemeenschappen in en buiten de pluim. Er werd een relatie gezien tussen dominante redox processen en aangetroffen microorganismen.

Betaproteobacteria waren sterk dominant. Echter, de *Betaproteobacteria* (*Acidovorax, Rhodoferax*) in de ijzerreducerende pluim verschilden aanzienlijk van deze aangetroffen stroomopwaarts van de vuilstort (*Gallionella, Azoarcus*). Leden van de ijzerreducerende familie *Geobacteraceae* droegen sterk bij aan de microbiële gemeenschap in de ijzerreducerende pluim, tot zo'n 25% van het totaal aantal bacteriën.

Geobacteraceae zijn ook veel voorkomend in andere ijzerreducerende locaties in de ondergrond die rijk zijn aan koolstofbronnen. Tot op heden is alleen voor leden van de Geobacters en *Rhodoferax* het vermogen tot de afbraak van mono-aromaten onder ijzerreducerende condities vastgesteld. Daarom zijn, op basis van de observaties beschreven in hoofdstuk 2, de relaties tussen samenstelling van de *Geobacteraceae* gemeenschap, graad van vervuiling, en snelheid van afbraak van vervuiling, in meer detail onderzocht voor de aquifer vervuild door de Banisveld vuilstort (**Hoofdstuk 3**). We namen een aanzienlijk verschil waar tussen de pluim en het onvervuilde deel van de aquifer in *Geobacter* gemeenschap samenstelling (op basis van cultivatie-onafhankelijke 16S rRNA gen gebaseerde DGGE fingerprinting en sequensen). Dit suggereert dat de vervuiling voor bepaalde Geobacters heeft geselecteerd. DGGE fingerprints van vervuilde grondwater genomen dichtbij de vuilstort (6-39 m afstand) groepeerden tezamen na numerieke analyse, terwijl de fingerpints van minder vervuild grondwater, dat meer stroomafwaarts werd genomen, niet in dezelfde groep vielen. De waargenomen hoge diversiteit in *Geobacteraceae* duidt op een grote genetische poel van Geobacters in de aquifer. Meer Geobacters, met andere fysiologische eigenschappen zijn mogelijk aanwezig, wat mogelijk inhoudt dat meer organische stoffen kunnen worden afgebroken. Een aantal banden in de DGGE profielen waren indicatief voor redox proces of de mate van vervuiling. De intensiteit van sommige vervuiling-indicatieve banden komt overeen met het deel van de aquifer waar organisch materiaal relatief het snelst wordt omgezet.

Hoewel moleculaire technieken een snel inzicht geven in welke microorganismen aanwezig zijn, leidt deze informatie vaak niet tot inzicht in de fysiologische eigenschappen van de aangetroffen microorganismen. Om ons inzicht in de natuurlijke degradatie van vuilstort percolaat onder ijzerreducerende condities verder te verbeteren, zijn ijzer reducerende consortia en isolaten van de aquifer vervuild door de Banisveld vuilstort, geïsoleerd (**Hoofdstuk 4**). IJzerreduceerders zoals *Serratia*, *Clostridium*, *Geobacter* en *Rhodoferax* spp werden geïsoleerd. Dit geeft aan dat naast *Geobacte*r er nog andere ijzerreducerende microorganismen aanwezig zijn. Echter het totaal aantal ijzer reducerende organismen dat kon worden gekweekt was laag, minder dan 110 per gram sediment. Hoewel wordt beweerd dat Geobacters, in tegenstelling tot veel andere microorganismen, makkelijk kunnen worden gekweekt, bleek het niet mogelijk de soort, welke een dominante band in DGGE fingerprints gaf en dominant was in kloon-banken (hoofdstuk 2 en 3), te isoleren. Dit ondanks het gebruik van een groot aantal verschillende condities voor incubatie (verschillende pHs, temperaturen, bronnen van koolstof en ijzer). Wel konden andere *Geobacter* soorten worden verrijkt en geïsoleerd.

Fysiologische aspecten van *Geobacteraceae* werden bestudeerd door *G. metallireducens* te cultiveren in een retentostat (continue culture met biomassa retentie) die de natuurlijke condities nabootst met een zeer lage groeisnelheid (0.008 h^{-1} en lager) (**Hoofdstuk 6**). De maximum groeiopbrengst was 0.05 – 0.09 C-mol biomassa per C-mol acetaat. De ijzerreducerende capaciteit van *G. metallireducens* was vergelijkbaar met die van *Shewanella putrefaciens*, de hoogste initiële ijzerreductie snelheid werd waargenomen met citraat-gechelateerd Fe (III) ($7x10^{-9}$ $\mu mol^{-1}cell^{-1}$ h^{-1}), de snelheden met amorf ijzerhydroxide en kristallijn hematiet waren 40 en 1500 maal lager, respectievelijk. De waargenomen zeer lage onderhoudsenergie (de laagste ooit gerapporteerd voor een bacterie) en het vermogen om direct alternatieve elektronenacceptoren te kunnen omzetten, zonder dat nieuwe enzymen moeten worden aangemaakt, geven (aanvullende en/of alternatieve) verklaringen voor het algemeen en dominant voorkomen van Geobacters onder ijzerreducerende condities.

Vergeleken met aquifers (hoofdstuk 2, 3 en 4), hebben ondiepe sedimenten in estuaria, zoals de Schelde, unieke en contrasterende biogeochemische eigenschappen, zoals hoge ruimtelijke (met diepte, op centimeter schaal) en tijdelijke dynamica in redox processen. Net als de aquifer vervuild door de Banisveld vuilstort, wordt ook de Schelde zwaar beïnvloed door antropogene activiteiten. Om het vermogen voor enzymatische ijzerreductie voor twee locaties in het Schelde stroomgebied, de zoetwater locatie Appels (België) en het brakke Waarde (Nederland). te bepalen zijn geochemische metingen op veldschaal gecombineerd met experimenten in het laboratorium naar de ijzerreducerende microorganismen. De microbiële gemeenschappen waren complex voor beide locaties. IJzerreducerende *Geobacter*, *Shewanella*, *Geothrix*, *Anaeromyxobacter* en *Alkaliphilus* soorten werden aangetroffen maar waren niet dominant aanwezig (minder dan 1% van de totale gemeenschap) in beide locaties. *Ralstonia* en *Clostridium* soorten, waarvan reincultures werden geïsoleerd, zijn dominante ijzerreduceerders in de Schelde. De hoge aanwezigheid van diverse types ijzerreduceerders (4.6×10^6, 2.4×10^5 cellen g^{-1} sediment voor Appels en Waarde, respectievelijk), de flexibiliteit van de ijzerreduceerders ten opzichte van variaties in pH en temperatuur, hun vermogen om alternatieve elektronacceptoren en donoren te verbruiken, en de aanzienlijke Fe (III) bio-beschikbaarheid, zijn sterke aanwijzingen dat er een aanzienlijk vermogen voor enzymatische ijzerreductie aanwezig is. De dynamiek en diversiteit in elektronendonoren en bijvoorbeeld de vorming van bio-beschikbaar Fe (III) (Fe-fosfaat) zou kunnen hebben geleid tot de ontwikkeling van een hoge fysiologische diversiteit in ijzerreduceerders en kan hebben geselecteerd tegen *Geobacteraceae*.

In hoofdstuk 7 worden de resultaten van de voorafgaande hoofdstukken besproken in relatie tot drie aspecten: (i) de rol van Geobacters in de biodegradatie van aromatische stoffen onder ijzerreducerende condities, (ii) de samenstelling van ijzerreducerende gemeenschappen in relatie tot hun omgeving, (iii) ijzerreductie in het kader van ecosysteem functioneren.

De aanwezigheid van sommige *Geobacte*r phylotypes is duidelijk gerelateerd aan de afbraak van organisch materiaal, zoals aromaten, in de aquifer vervuild door de Banisveld vuilstort, wat erop lijkt te duiden dat *Geobacter* phylotypes zijn betrokken bij de afbraak van aromaten. Behalve de dominante Geobacters, zijn ook ijzerreducerende *Serratia*, *Desulfitobacterium*, *Clostridium* en *Rhodoferax* aangetroffen. Dit duidt op de aanwezigheid van een poel van functioneel redundante (voor wat betreft ijzerreductie) microorganismen, welke mogelijk ook betrokken zijn bij de anaërobe afbraak van vervuiling. Daarom zouden naast moleculaire methoden die zich richten op 16S rRNA genen, zoals toegepast in dit onderzoek, ook methoden voor de detectie van genen betrokken bij de anaërobe afbraak van aromatische stoffen moeten worden toegepast in het vaststellen van het vermogen tot en daadwerkelijk plaatsvinden van *in situ* afbraak. Andere opties voor *in situ* bioremediatie zoals de toevoeging van chelatoren of elektron shuttles, worden ook besproken.

De samenstelling van ijzerreduceerde gemeenschappen wordt verondersteld te zijn gerelateerd aan de omgeving waarin zij voorkomen. *Geobacters* worden wereldwijd aangetroffen in de ijzerreducerende ondergrond met neutrale pH, met name als ook koolstof bronnen, b.v. door vervuiling, beschikbaar zijn. De factoren die hiertoe zouden bijdragen relateren aan hun genetische en fysiologische eigenschappen. Echter, *Geobacter* en ook *Shewanella*, model stammen in onderzoek naar ijzer reductie, zijn niet dominant in zure omgevingen en ook niet in de Schelde, zoals beschreven in dit proefschrift, hoewel deze een neutrale pH heeft. De aanwezigheid en beschikbaarheid van diverse elektronendonoren, als ook ijzer en andere elektronen acceptoren in de Schelde zijn mogelijk de oorzaak voor de fylogenetische diversiteit en fysiologische flexibiliteit daar aangetroffen.

IJzerreductie is een van de delen in ecosysteem functioneren, daar dit proces sterk verbonden is met het lot van het organisch materiaal en de cycli in metaal en nutriënten. Om ecologische processen beter te kunnen begrijpen, zou het gehele systeem (structuur van de gemeenschap, activiteiten van individuele leden en interacties tussen de verschillende leden, als ook hun omgeving (de chemische en fysische structuur)) moeten worden beschouwd voor een beter begrip van ijzerreductie.

论 文 总 结

本研究旨在深入了解微生物介入的铁还原反应在环境中的作用.我们研究了两种完全不同的环境中铁还原微生物群体的组成及其功能，即被垃圾处理厂污染的含水土层和河水入海口处的沉积泥层。 在本试验中,通过实地的水文地球化学测量和实验室鉴定来完成对铁还原微生物组成及其功能的了解. 通过对从环境中富积和分离出的铁还原微生物核糖体(16S rRNA)和生理学的分析，进而获得对该类微生物详尽的知识. 同时,对严格厌氧模式铁还原菌 *Geobacter metallireducens* 分别在封闭静止条件下(batch)和连续,可保留生物量的培养条件下(retentostat)进行生理学研究,以更深入地了解该类微生物的特性。

在第一章的总引言中，从八个方面讨论了微生物介入的铁还原现象的研究背景：(i) 非生物和生物铁还原；(ii) 生物铁还原的重要性;(iii) 生物铁还原发生的地点和方式;(vi)已知能够还原三价氧化铁的纯培养微生物;(v)微生物还原三价氧化铁的机理;(vi)氧化铁的生物利用率和铁还原的关系;(vii)在地表以下通常存在的电子供体；(viii) 从分子生物学水平对铁还原环境中的微生物群体的评估。

在被以堆积法处理垃圾污染的地下水中,铁还原现象是常遇到的主要的氧化还原过程. 在**第二章**里,对被荷兰的班尼斯夫尔 (Banisveld, The Netherlands) 垃圾处理厂污染的地下水微生物群体结构和水化学的关系进行了阐述。在未污染的参照地点,两种氧化还原过程被观察到:一种是在污染区域以上以反硝化作用为主，另一种是在污染区域及其以下以铁还原作用为主。 污染有机物的降解发生在污染区域,该区以铁还原为主要的氧化还原作用。以核糖体基因(16Sr RNA)为基础的变性梯度凝胶电泳 (DGGE) 量化分析揭示，污染和非污染区域的细菌和古细菌的核糖体基因型群体结构明显不同。变性梯度凝胶电泳分析与核糖体基因型分析相吻合。 我们观察到占优势的氧化还原作用具有相应的微生物分子生物学特征。在建立的克隆库中,属于β-Proteobacteria 的基因型占主导地位。然而,从以铁还原作用为主的污染区域获得的，同属于β-*Proteobacteria* 的 *Acidovorax, Rhodoferax*,明显地不同于从位于污染源上游的非污染区获得的基因型 *Gallionella* 和 *Azoarcus*。在污染的,铁还原为主要作用的区域,隶属于 *Geobacteraceae* 的基因型占据较大的比例，达 25%的细菌总量计数。

在其它碳源丰富，以铁还原作用为主的土层下，隶属于 *Geobacteraceae* 的微生物种占据微生物群体中的大多数。 迄今为止,在铁还原作用下只有来自 Geobacters 和 *Rhodoferax* 的微生物能够降解单环芳香族化合物。 因此,第二章中的发现启示我们进行**第三章**的研究：即探讨铁还原微生物 *Geobacteraceae* 群体,污染程度和污染物降解之间的详尽关系。通过以核糖体基因(16Sr RNA)为基础的变性梯度凝胶电泳和 DNA 测序分析,观察到来自污染区域 *Geobacter* 的种类明显地不同与来

自非污染区，意旨污染条件对 *Geobacter* 种具有选择性。变性梯度凝胶电泳分析显示来自距离污染源 6-39 米区域的 *Geobacter* 基因型种明显地区别于非污染区 *Geobacter* 基因型种类. 而且，高多样性 *Geobacteraceae* 基因型种类说明在班尼斯夫尔(Banisveld, The Netherlands)垃圾处理厂地下水中存在一个大的铁还原菌 *Geobacter* 库。多样性的 *Geobacter* 种类意示着不同的生理特性，这使得更广泛的有机化合物在该研究区域可被降解.某些凝胶电泳带在统计上明显地与主导氧化还原作用或污染程度相关联。 凝胶电泳带的存在及其强弱与有机污染物和活性可溶性有机物的高衰减率呈正相关性。

分子生物学分析可迅速地测定微生物的存在，然而无法提供其潜在及真正的生理功能. 在**第四章**中,为了进一步加深对铁还原条件下垃圾处理厂沥渗物自然衰减的了解,我们利用污染的地下含水土层构建了铁还原多菌富积培养物和分离出纯培养铁还原菌。这些铁还原细菌是 *Serratia, Clostridium,* and *Geobacter spp.* 而 *Rhodoferax sp.* 在用连续稀释接种物方法建立的培养物中为优势菌。 这说明在被垃圾处理厂沥渗物污染的地下含水土层中孕藏着多样性的铁细菌，而不仅仅是 *Geobacter* 菌. 在被污染的沉积物中，可培养的铁细菌每克含 80-140 个,是非污染处的五倍。 虽然常有报道称 *Geobacter* 菌较其它环境微生物容易培养，然而，尽管我们采用了不同的培养温度，酸碱度,不同形式的三价铁作为电子受体和不同的碳源,在凝胶电泳和核糖体基因克隆分析中观察到的主导优势菌 *Geobacter*（如在第一，二章中描述）并未被富积和分离出来。但是我们富积和分离到了其它 *Geobacter* 菌株。

在**第六章**中,严格厌氧摸式菌 *G. metallireducens* 在模拟自然环境中的生长，即在可滞留生物量的连续培养装置中，和在非常低的生长速率 ($0.008h^{-1}$) 的条件下，对有关 *Geobacteraceae* 生理方面进行了研究。 该菌的最大产量为介于 0.05 和 0.09 C-mol 生物量/ C-mol 乙酸。 *G. metallireducens* 的铁还原容量与 *Shewanella putrefaciens* 相当，以柠蒙酸三价铁为电子受体的最大起始铁的还原率为 $7x10^{-9}$ mmol $cell^{-1}$ h^{-1} ，分别是无定形三价氧化铁 (ferrihydrite) 和结晶体纳米型的氧化铁(nanohematite) 的 40 和 1500 倍。 我们测到 *G. metallireducens* 维持生存能量的需求非常低，是迄今报道的异养型细菌中最低的。 极低的生存维持能量需求，以及从反映器中收获的细胞具有应用其他的电子受体而无须合成新蛋白的能力，这些发现可对为何 Geobacters 普遍存在，而且作为优势菌存在于许多铁还原地下环境中提供了生理学上的补充解释。

与第二，三和四章有关地下环境相比，浅表入海口沉积层具有独特的生物地质化学特性，正如本研究的地点斯浩得入海口 (Scheldt)，氧化还原作用随空间 (深度)，和时间的显著变化。 与班尼斯夫尔地下水层类似，斯浩得入海口的生态系统严重的被人类的活动影响，即由于排入复杂的有机物所导致. **第五章**中，为了探讨

微生物介入的铁还原现象在入海口的潜在作用，我们选择该处的淡水区域 Appels (位于比利时境内)，和咸水区域 Waarde (位于荷兰境内)，进行野外实地的地球化学测量和实验室的相关微生物研究。 在两个研究地点，铁还原细菌群体的结构均较复杂。 我们发现了隶属于 *Geobacter, Shewanella, Geothrix, Anaeromyxobacteria* 和 *Alkaliphilus* 属的铁细菌，但是这些菌并非占主导地位，仅占细菌计数的 1%。来自 *Ralstonia* 和 *Clostridium* 属的纯培养细菌则在斯浩得入海口(Scheldt) 呈现为主要的铁细菌。 另外，一些占较小比例的菌分别来自 *Bacteroidetes* (22% 在淡水区域的 Appels，和 20% 咸水区域 的 Waarde) 和 *Actinobacteria* (5%，淡水区域-Appels) 或 *Fusobacteria* (7% 咸水区域-Waarde) 。 多样性和大量 (在 Appels 和 Waarde 每克沉积物分别含 4.6×10^6，2.4×10^5 个细菌) 存在的铁细菌，及其广泛的对酸碱度和温度的适应力，能够灵活地利用不同的电子共体和受体，以及环境中大量存在的可被生物利用的三价铁，这些现象强有力地说明微生物介入的铁还原作用存在。 多变的，多种类的电子供体和电子受体的氧化还原循环(如磷酸铁的形成)可能导致生理性状不同的铁细菌共存，这也可能是来自 *Geobacteraceae* 的严格厌氧菌无法占优势的原因之一。

第七章从三个方面讨论了前面几章的结果，(1) 在铁还原条件下,Geobacters 在芳香族化合物生物降解中的作用；(2) 铁还原细菌群体的组成与环境条件的关系；(3) 铁还原现象作为生态系统中的功能之一。

在被以荷兰班尼斯夫尔处理厂污染的地下水中，某些来自 *Geobacter* 的种类明显地与可溶性有机碳，苯，甲苯， 乙苯和二甲苯的衰减率相关，说明某些 *Geobacter* 参与了芳香族化合物的降解。 除了优势菌 Geobacters，来自 *Serratia, Desulfitobacterium, Clostridium* 和 *Rhodoferax* 的铁还原菌同样从污染区域被富积或分离到。 这种现象说明该处存在着一个同功能 (即铁还原作用) 的微生物群体，这些铁还原菌可能参与了厌氧条件下的芳香族生物降解。 除了基于核糖体 16Sr RNA 的技术，用于检测和追踪功能基因的技术应当完善。只有在这种条件下，引入外源活性微生物用于生物矫正方为一种可行的措施. 另一方面，对有关原位生物矫正的一些措施作了进一步的讨论，如添加敖合物或电子传递媒介体以提高生长限制因子三价氧化铁的生物利用率。

铁还原细菌的群体组成被认为与环境条件相关。 在中性条件下，Geobacters 会常常在以铁还原作用为主，被石油，垃圾沥渗物，以及金属污染的地下层中遇到。 其遗传和生理特性决定了为什么在此环境中常遇到 Geobacters. 来自 *Geobacter* 和 *Shewanella* 的菌常被作为研究铁还原现象的模式菌，然而他们在酸性及中性的斯浩得入海口(Scheldt)并不是主要的铁还原菌. 可利用的，多样性的电子供体和三价氧化铁的电子受体导致遗传上和生理上多样性的铁细菌的存在，如

Ralstonia, Clostridium, Geobacter, Shewanella, Geothrix, Anaeromyxobacter 和 *Alkaphilius*。

铁还原作为生态系统功能中的一部分，紧密地与有机物的降解，金属和营养物的循环相关。 为了更好地了解生态学的过程，应把该系统视为一个整体，即综合研究微生物群体结构，个体作用，不同个体之间相互作用，与环境之间的作用，以及它们在环境中的物理化学作用，这样可对铁还原反应在生态系统中的作用有更深入地理解。

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Dr. Henk van Verseveld
Alps, France. 2003

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